

University of Alberta

**MOLECULAR MECHANISMS MEDIATING LY49 SPECIFICITY FOR CLASS I  
MAJOR HISTOCOMPATIBILITY MOLECULES**

by

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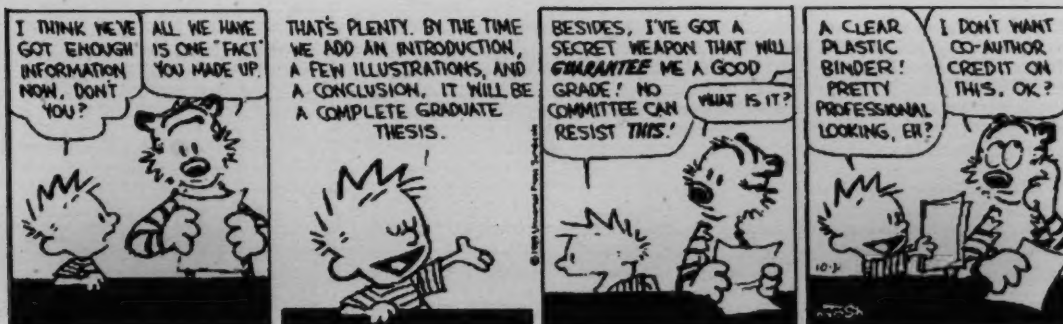
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*To all of you who participated in and brought light to the adventure ...*

## ABSTRACT

Murine Ly49 receptors exhibit allele specific recognition for their polymorphic MHC I ligands. However, the site of interaction on MHC I consists of highly conserved solvent exposed amino acids in both rat and mouse alleles, leaving it unclear how allele specificity occurs. We identify a rat classical MHC I molecule, RT1-A1<sup>c</sup>, as a xenogeneic ligand for the mouse inhibitory Ly49G and activating Ly49W receptors that allows us to identify motifs conserved between these two species that mediate xeno-recognition. The motif shared between ligands for Ly49G, W and the rat inhibitory receptor Ly49i2, which is the syngeneic receptor for RT1-A1<sup>c</sup>, is a common supertype. MHC I molecules typically belong to one of about nine or ten different supertypes defined by the residues in the bound peptide that act to anchor the peptide into specific pockets of the MHC I peptide-binding groove. Through mutagenesis, we show that the supertype-defining B-pocket of RT1-A1<sup>c</sup> specifically influences recognition by syngeneic and xenogeneic Ly49. Our findings suggest that the supertype-defining B-pocket dictates the conformation of conserved solvent exposed residues at the Ly49 interaction site below both directly and through the binding of specific peptide anchor residues. Additional mutagenesis studies indicate that both rat and mouse Ly49/MHC I allele combinations rely on solvent exposed residues at three site 2 subsites. These include conserved interactions with residues below the more conserved anchor binding F-pocket as well as differential interactions with residues below the polymorphic anchor binding B-pocket and with residues in the  $\alpha 3$  domain of MHC I. These mutagenesis studies demonstrate that both rat and mouse Ly49 interact at site 2 of MHC I and allow us to propose a

comprehensive model for how polymorphic Ly49 mediate allele specificity for MHC I at a highly conserved interaction site based on supertype defined conformations that are shared across divergent species. This may allow NK cells to sense whether expression of specific MHC I superotypes capable of antigen presentation to T cells are expressed, and if lacking, would target a cell for elimination, suggesting a supertype-mediated link between innate and adaptive immunity.

## TABLE OF CONTENTS

| CHAPTER                                                                                                                                                         | PAGE |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| <b>I. INTRODUCTION</b>                                                                                                                                          |      |
| A. NK Cells and Their Function.....                                                                                                                             | 1    |
| B. NK Cell Receptors and Their Ligands .....                                                                                                                    | 4    |
| <i>Receptors of the LRC</i> .....                                                                                                                               | 4    |
| <i>Receptors of the NKC</i> .....                                                                                                                               | 6    |
| C. The Murine Ly49 Receptor Repertoire .....                                                                                                                    | 8    |
| D. Class I MHC.....                                                                                                                                             | 11   |
| E. Allele Specific Ly49 Recognition of Class I MHC.....                                                                                                         | 15   |
| F. Background and Rationale.....                                                                                                                                | 18   |
| G. References.....                                                                                                                                              | 26   |
| <br><b>II. THE RAT RT1-A1<sup>C</sup> MHC MOLECULE IS A XENOGENEIC LIGAND<br/>RECOGNIZED BY THE MOUSE ACTIVATING LY-49W AND INHIBITORY<br/>LY-49G RECEPTORS</b> |      |
| A. Introduction.....                                                                                                                                            | 50   |
| B. Materials and Methods                                                                                                                                        |      |
| <i>Animals</i> .....                                                                                                                                            | 52   |
| <i>Hybridomas and antibodies</i> .....                                                                                                                          | 52   |
| <i>Cell lines</i> .....                                                                                                                                         | 52   |
| <i>Mutagenesis of Ly-49W and Ly-49P</i> .....                                                                                                                   | 53   |
| <i>RNA preparation and cDNA synthesis</i> .....                                                                                                                 | 53   |
| <i>Generation of YB2/0 target cells expressing rat class I MHC-EGFP<br/>    fusion proteins</i> .....                                                           | 53   |
| <i>Flow cytometric analysis</i> .....                                                                                                                           | 54   |
| <i>Generation of Con A T cell blasts</i> .....                                                                                                                  | 54   |
| <i>Cytotoxicity assays</i> .....                                                                                                                                | 54   |



### C. Results

|                                                                                                                                                                                                                    |    |
|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <i>Con A blasts from the PVG rat strain are recognized by Ly-49W and the ectodomain of the BALB/c allele of Ly-49G</i> .....                                                                                       | 55 |
| <i>RT1<sup>av/c</sup> intra-MHC recombinants identify the classical class I region of the RT1<sup>c</sup> haplotype as essential for recognition by Ly-49W and Ly-49WG<sup>BALB/c</sup></i> .....                  | 57 |
| <i>The classical rat class I MHC molecule, RT1-A1<sup>c</sup>, is a xenogeneic ligand for the murine Ly-49W and Ly-49G<sup>BALB/c</sup> receptors</i> .....                                                        | 58 |
| <i>Mutagenesis of Ly-49P <math>\beta</math>4-<math>\beta</math>5 loop residues to corresponding residues of Ly-49W and Ly-49G<sup>BALB/c</sup> receptors confers specificity for a ligand in the PVG rat</i> ..... | 60 |

|                     |    |
|---------------------|----|
| D. Discussion ..... | 61 |
|---------------------|----|

|                           |    |
|---------------------------|----|
| E. Acknowledgements ..... | 66 |
|---------------------------|----|

|                                      |    |
|--------------------------------------|----|
| F. Author Contribution to Data ..... | 66 |
|--------------------------------------|----|

|                     |    |
|---------------------|----|
| G. References ..... | 76 |
|---------------------|----|

## III. CROSS-SPECIES DEPENDENCE OF LY49 RECOGNITION ON THE SUPERTYPE DEFINING B-POCKET OF A CLASS I MHC MOLECULE

|                       |    |
|-----------------------|----|
| A. Introduction ..... | 84 |
|-----------------------|----|

### B. Materials And Methods

|                                                   |    |
|---------------------------------------------------|----|
| <i>Hybridomas and monoclonal antibodies</i> ..... | 86 |
| <i>Cell lines</i> .....                           | 86 |
| <i>Cloning and transfection of Ly49i2</i> .....   | 87 |
| <i>Mutagenesis of RT1-A1<sup>c</sup></i> .....    | 87 |
| <i>Flow cytometric analysis</i> .....             | 87 |
| <i>Cytotoxicity assays</i> .....                  | 88 |
| <i>Peptide elutions</i> .....                     | 88 |

### C. Results

|                                                                                                                                                                       |    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| <i>RT1-A1<sup>c</sup> recognition by a syngeneic rat inhibitory receptor and xenogeneic mouse inhibitory and activating receptors expressed on RNK-16 cells</i> ..... | 89 |
| <i>Xenogeneic mouse and syngeneic rat Ly49 recognition of RT1-A1<sup>c</sup> follows a pattern similar to mouse syngeneic recognition</i> .....                       | 90 |

|                                                                                                                                                                                                      |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <i>Xenogeneic and syngeneic Ly49 recognition is sensitive to changes in B-pocket residues of RT1-A1<sup>c</sup></i> .....                                                                            | 91  |
| <i>Xenogeneic and syngeneic recognition of RT1-A1<sup>c</sup> is not sensitive to alterations of non B-pocket residues along the length of the peptide-binding groove</i> .....                      | 93  |
| <i>B-pocket mutants that disrupt syngeneic recognition are loaded with fewer peptides having proline at P2 while xenogeneic recognition can be disrupted without a change in P2 preference</i> ..... | 94  |
| D. Discussion .....                                                                                                                                                                                  | 95  |
| E. Acknowledgments .....                                                                                                                                                                             | 102 |
| F. Author Contribution to Data .....                                                                                                                                                                 | 102 |
| G. References .....                                                                                                                                                                                  | 109 |

#### IV. RAT AND MOUSE LY49 RECEPTORS USE VARIABLE RESIDUES AT THREE SUBSITES AT SITE 2 FOR ALLELE SPECIFIC RECOGNITION OF CLASS I MHC MOLECULES

|                                                                                                                                                                                                                        |     |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| A. Introduction .....                                                                                                                                                                                                  | 115 |
| B. Materials and Methods                                                                                                                                                                                               |     |
| <i>Hybridomas and monoclonal antibodies</i> .....                                                                                                                                                                      | 118 |
| <i>Cell lines</i> .....                                                                                                                                                                                                | 118 |
| <i>Cloning and transfection of Ly49i2</i> .....                                                                                                                                                                        | 118 |
| <i>Mutagenesis of MHC I</i> .....                                                                                                                                                                                      | 119 |
| <i>Flow cytometric analysis</i> .....                                                                                                                                                                                  | 119 |
| <i>Cytotoxicity Assays</i> .....                                                                                                                                                                                       | 120 |
| C. Results                                                                                                                                                                                                             |     |
| <i>Ly49G recognition of H-2D<sup>d</sup> is disrupted by mutagenesis of single residues articulating with anchor binding pockets at subsite B whereas Ly49i2 recognition of RT1-A1<sup>c</sup> is unaffected</i> ..... | 120 |
| <i>Ly49i2 and Ly49G both exhibit sensitivity to alterations in solvent exposed residues below and articulating with the F-pockets of RT1-A1<sup>c</sup> and H-2D<sup>d</sup> at subsite F</i> .....                    | 123 |

|                                                                                                                                                                                                                                                 |     |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| <i>Specific single amino acid changes at subsite-C in the MHC I <math>\alpha 3</math> domain, can completely abolish Ly49G recognition of H-2D<sup>d</sup>, whereas Ly49i2 recognition of RT1-A1<sup>c</sup> is relatively unaffected</i> ..... | 124 |
| <i>Ly49i2 recognition of RT1-A1<sup>c</sup> is sensitive to combined alanine mutations at two different subsites</i> .....                                                                                                                      | 125 |
| <i>Ly49i2 recognition of RT1-A1<sup>c</sup> is sensitive to double alanine mutations at each individual subsite</i> .....                                                                                                                       | 126 |
| D. Discussion .....                                                                                                                                                                                                                             | 128 |
| E. Acknowledgments .....                                                                                                                                                                                                                        | 137 |
| F. Author Contribution to Data .....                                                                                                                                                                                                            | 137 |
| G. References .....                                                                                                                                                                                                                             | 148 |

## V. DISCUSSION AND CONCLUSION

|                                                          |     |
|----------------------------------------------------------|-----|
| A. Summary of Contributions .....                        | 155 |
| B. Xeno-transplantation .....                            | 158 |
| C. Orphan Ly49 Receptors .....                           | 161 |
| <i>Viral Ligands</i> .....                               | 161 |
| <i>Rat Ly49 Ligands</i> .....                            | 163 |
| D. Convergent Evolution .....                            | 164 |
| <i>Ly49 Receptor Surveillance and CTL Function</i> ..... | 165 |
| <i>Functional Divergence in Rodents</i> .....            | 169 |
| E. Future Directions .....                               | 171 |
| <i>Xeno-transplantation Studies</i> .....                | 171 |
| <i>Peptide Loading Studies</i> .....                     | 172 |
| <i>Rat Studies</i> .....                                 | 174 |
| F. Conclusion .....                                      | 175 |
| G. References .....                                      | 179 |

## LIST OF TABLES

| Table | Description                                                                                    | Page |
|-------|------------------------------------------------------------------------------------------------|------|
| 3-1   | P2 residues of peptides eluted from wild-type and mutant RT1-A1 <sup>c</sup><br>molecules..... | 108  |



## LIST OF FIGURES

| Figure | Description                                                                                                                                                                                                            | Page |
|--------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| 1-1    | NK cell receptor genes in the LRC and NKC of human, mouse and rat.....                                                                                                                                                 | 22   |
| 1-2    | Activating and inhibitory Ly49 structure and signaling pathways.....                                                                                                                                                   | 23   |
| 1-3    | Comparison of the human, mouse and rat MHC Complexes.....                                                                                                                                                              | 24   |
| 1-4    | Crystal structures depicting the H-2D <sup>d</sup> MHC I molecule and its association<br>at two distinct sites with Ly49A.....                                                                                         | 25   |
| 2-1    | Xenogeneic recognition of Con A blasts from the PVG rat strain by the mouse<br>Ly-49W activating receptor and Ly-49WG <sup>BALB/c</sup> activating chimera.....                                                        | 67   |
| 2-2    | The rat major histocompatibility complex and haplotypes of selected rat<br>strains.....                                                                                                                                | 68   |
| 2-3    | Lysis of RT1 <sup>av1/c</sup> intra-MHC recombinant targets identifies the classical class<br>I region of the RT1 <sup>c</sup> haplotype as essential for recognition by Ly-49W and<br>Ly-49WG <sup>BALB/c</sup> ..... | 69   |
| 2-4    | Expression of rat MHC-EGFP fusion proteins on transfected rat YB2/0 cells.....                                                                                                                                         | 70   |
| 2-5    | Ly-49W and the Ly-49WG <sup>BALB/c</sup> chimera recognize RT1-A1 <sup>c</sup> , a classical<br>class I MHC molecule from the PVG rat strain.....                                                                      | 71   |
| 2-6    | The inhibitory Ly-49G <sup>BALB/c</sup> receptor recognizes RT1-A1 <sup>c</sup> .....                                                                                                                                  | 72   |
| 2-7    | Mutagenesis of the Ly-49P $\beta$ 4- $\beta$ 5 loop to express the Ly-49W motif DCGK<br>confers specificity for a ligand in the PVG rat strain.....                                                                    | 73   |
| 2-8    | The Ly-49P and Ly-49W $\beta$ 4- $\beta$ 5 loop mutants recognize RT1-A <sup>c</sup> encoded<br>ligand(s).....                                                                                                         | 74   |
| 2-9    | Dendrogram comparing the nucleotide sequences of five known and ten<br>predicted rat Ly-49 receptors to eight mouse Ly-49 receptors.....                                                                               | 75   |
| 3-1    | The rat classical class I MHC molecule, RT1-A1 <sup>c</sup> , is a xenogeneic ligand for<br>mouse Ly49G <sup>BALB/c</sup> and Ly49W and a syngeneic ligand for Ly49i2.....                                             | 103  |
| 3-2    | Xenogeneic and syngeneic Ly49 recognition of RT1-A1 <sup>c</sup> is at site 2.....                                                                                                                                     | 104  |

|     |                                                                                                                                                                                                                                     |     |
|-----|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----|
| 3-3 | Xenogeneic and syngeneic Ly49 recognition of RT1-A1 <sup>c</sup> is sensitive to alterations in the anchor residue binding B-pocket of the MHC I molecule.....                                                                      | 105 |
| 3-4 | Xenogeneic and syngeneic Ly49 recognition of RT1-A1 <sup>c</sup> is not sensitive to alterations in the peptide-binding groove outside of the anchor binding B-pocket of the MHC I molecule.....                                    | 106 |
| 3-5 | Alterations in the polymorphic and supertype defining B-pocket of RT1-A1 <sup>c</sup> disrupts interaction with Ly49 receptors, possibly by altering the conformation of solvent exposed residues at site 2.....                    | 107 |
| 4-1 | Designation of site 2 subsites and MHC I recognition by Ly49i2 and Ly49G.....                                                                                                                                                       | 138 |
| 4-2 | Mutagenesis of single residues at three MHC I subsites differentially effect Ly49i2 and Ly49G recognition of their ligands.....                                                                                                     | 139 |
| 4-3 | Ly49i2 recognition of RT1-A1 <sup>c</sup> is unaffected by single B-subsite mutants whereas Ly49G recognition of H-2D <sup>d</sup> is disrupted by single mutants beneath the anchor binding B- and C-pockets at the B-subsite..... | 140 |
| 4-4 | Single residue mutagenesis at the F-subsite completely disrupts Ly49G recognition of H-2D <sup>d</sup> and partially disrupts Ly49i2 recognition of RT1-A1 <sup>c</sup> when at residue D122.....                                   | 141 |
| 4-5 | RT1-A1 <sup>c</sup> recognition by Ly49i2 is relatively unaffected by single C-subsite mutations, while Ly49G recognition of H-2D <sup>d</sup> is sensitive to specific C-subsite point mutations.....                              | 142 |
| 4-6 | Recognition of RT1-A1 <sup>c</sup> by Ly49i2 is completely disrupted by combined mutations at any two subsites.....                                                                                                                 | 143 |
| 4-7 | Ly49i2 recognition of RT1-A1 <sup>c</sup> relies on each individual subsite at site 2, relying on a Ly49A/C hybrid-type residue usage at the C-subsite.....                                                                         | 144 |
| 4-8 | Double mutants at each subsite show that Ly49i2 recognition of RT1-A1 <sup>c</sup> is completely dependent on subsite-F, less so on residues at the B-subsite and occurs in a unique manner at the C-subsite.....                   | 145 |

|             |                                                                                                                                                                                                                                                                                                          |            |
|-------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <b>4-9</b>  | Location of key residues discussed in this study that were previously identified as mediating recognition in the Ly49A/H-2D <sup>d</sup> and Ly49C/H-2K <sup>b</sup> co-crystals.....                                                                                                                    | <b>146</b> |
| <b>4-10</b> | Polymorphisms in the anchor binding pocket(s) of MHC I may affect Ly49 recognition through conformational changes in solvent exposed residues that articulate simultaneously with the anchor binding pocket(s) of MHC I and with conserved $\beta$ 2m residues that affect its orientation on MHC I..... | <b>147</b> |
| <b>5-1</b>  | Cartoon depiction of how conformational changes in the $\alpha$ 1 $\alpha$ 2 domains, particularly in the supertype-defining B-pocket, may alter MHC I recognition by allele specific Ly49 at a highly conserved interaction site.....                                                                   | <b>176</b> |
| <b>5-2</b>  | Creation of xenogeneic mixed chimeras.....                                                                                                                                                                                                                                                               | <b>177</b> |
| <b>5-3</b>  | T cell receptor (TCR) interaction with MHC I.....                                                                                                                                                                                                                                                        | <b>178</b> |

## LIST OF ABBREVIATIONS

|                  |                                                  |
|------------------|--------------------------------------------------|
| ADCC             | antibody dependent cellular cytotoxicity         |
| AICL             | activation induced C-type lectin                 |
| AIDS             | acquired immunodeficiency syndrome               |
| ALC              | allogeneic lymphocyte cytotoxicity               |
| APC              | antigen presenting cell                          |
| $\beta 2m$       | beta-2 microglobulin                             |
| CD               | cluster of differentiation                       |
| cDNA             | complementary deoxyribonucleic acid              |
| CDR              | complimentarity determining region               |
| CHO              | Chinese hamster ovary cells                      |
| Clr              | C-type lectin related molecule                   |
| CMV              | cytomegalovirus                                  |
| ConA             | concanavalin A                                   |
| $^{51}\text{Cr}$ | $\text{Na}^{51}\text{CrO}_4$                     |
| CRD              | carbohydrate recognition domain                  |
| CTL              | cytotoxic T lymphocyte                           |
| CTLD             | C-type lectin-like domain                        |
| DC               | dendritic cell                                   |
| EGFP             | enhanced green fluorescent protein               |
| E:T              | effector to target cell ratio                    |
| FACS             | fluorescence activated cell sorter               |
| FasL             | Fas ligand                                       |
| FCS              | foetal calf serum                                |
| FITC             | fluorescein isothiocyanate                       |
| G-CSF            | granulocyte-colony stimulating factor            |
| GM-CSF           | granulocyte macrophage-colony stimulating factor |
| GVHD             | graft versus host disease                        |
| GvL              | graft versus leukemia                            |
| HCMV             | human cytomegalovirus                            |
| HLA              | human leukocyte antigen                          |
| HSC              | haematopoietic stem cells                        |
| HSCT             | haematopoietic stem cell transplantation         |
| IFN              | interferon                                       |
| Ig               | immunoglobulin                                   |



|             |                                                                 |
|-------------|-----------------------------------------------------------------|
| IL          | interleukin                                                     |
| ILT2        | immunoglobulin-like transcript-2                                |
| ITIM        | immuno-receptor tyrosine based inhibitory motif                 |
| ITAM        | immuno-receptor tyrosine based activation motif                 |
| KIR         | killer cell immunoglobulin-like receptor                        |
| KLRF        | killer cell lectin-like receptor                                |
| LAIR        | leukocyte-associated immunoglobulin-like receptor               |
| LILR        | leukocyte immunoglobulin-like receptor                          |
| LLT1        | lectin-like transcript-1                                        |
| LRC         | leukocyte receptor complex                                      |
| LT $\alpha$ | lymphotoxin- $\alpha$                                           |
| MCMV        | murine cytomegalovirus                                          |
| M-CSF       | macrophage-colony stimulating factor                            |
| MHC         | major histocompatibility complex                                |
| MHC I       | class I MHC                                                     |
| MIP-1       | macrophage inflammatory protein-1                               |
| mRNA        | messenger ribonucleic acid                                      |
| NCR         | natural cytotoxicity receptor                                   |
| NKC         | natural killer gene complex                                     |
| NK cell     | natural killer cell                                             |
| NOD         | nonobese diabetic                                               |
| NOR         | non-obese resistant                                             |
| PIR         | paired immunoglobulin-like receptor                             |
| rADCC       | reverse antibody-dependent cell-mediated cytotoxicity           |
| Rae-1       | retinoic acid early-1 protein                                   |
| RANTES      | regulated upon activation, normal T cell expressed and secreted |
| SHIP        | SH2 domain containing inositol-5 phosphatase                    |
| SHP         | src homology 2 domain-containing tyrosine phosphatase           |
| TAP         | transporter for antigen presentation                            |
| TBI         | total body irradiation                                          |
| TCR         | T cell receptor                                                 |
| TI          | thymic irradiation                                              |
| TNF         | tumor necrosis factor                                           |
| TRAIL       | tumor necrosis factor-related apoptosis-inducing ligand         |
| ULBP        | UL16 binding protein                                            |

## CHAPTER I

### INTRODUCTION

#### A. NK Cells and Their Function

Natural killer cells (NK cells) are large granular lymphocytes (1) that, along with cells of the myeloid lineage such as monocytes/macrophages, dendritic cells (DC) and granulocytes, participate in mediating innate immune responses. In contrast to B and T lymphocytes, the mediators of adaptive immunity, NK cells can mediate spontaneous effector functions without prior antigen sensitization, do not undergo genetic rearrangement and somatic mutation of their recognition receptors and do not display memory for antigen. Developed in the bone marrow (2, 3), mature NK cells make up approximately 5-20% of peripheral lymphocytes in the spleen, liver and peripheral blood and are also found in smaller proportions in the thymus, lymph nodes and bone marrow (4). They function as killers, cytokine producers and regulators of adaptive immunity (5).

NK cell killing occurs via two main cytolytic pathways, either granule exocytosis (6) or through the activation of death receptors (7-9). The cytotoxic granules within NK cells contain perforin and a family of serine proteases known as granzymes, which are released by an attacking NK cell and act respectively to disrupt the cell membrane and to induce apoptosis of an engaged target cell (10). Death receptors on NK cells include Fas ligand (FasL), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and lymphotoxin- $\alpha$  (LT $\alpha$ ) (7-9), with some receptors, such as TRAIL, being found primarily on certain NK subsets or upon NK activation by cytokines such as IL-15 and IFN $\gamma$  (7). These receptors trigger apoptosis of target cells upon engagement with ligands containing cytoplasmic death domains and have important functions, including the control of tumor growth (11).

Cytokines and chemokines also have an essential role in NK effector functions. NK cells secrete GM-CSF, G-CSF, M-CSF, TNF $\alpha$ , IL-5, IL-10, IL-13, IFN $\gamma$ , (12) and numerous chemokines including MIP-1 and RANTES (13) in the regulation of hematopoiesis, tumor growth, inflammation, viral responses, and in priming effectors important for adaptive immunity. Equally, NK cells respond to a myriad of cytokines

including IL-2, IL-12, IL-15, IL-18, IL-21, and type I interferons affecting their development, activation, cytokine secretion, cytotoxicity and proliferation (4). In particular, NK cell responses are enhanced by interactions with DC, which present antigen to T cells in adaptive immunity, responding optimally upon DC stimulation through a combination of soluble mediators and cell-to-cell contact. This "cross-talk" between NK cells and DC is reciprocal with NK cells playing an important role in the regulation of DC function, by facilitating DC maturation when the NK to DC ratio is low and eliminating immature DC in situations where the NK to DC ratio is high, likely through the engagement of death receptors on the NK cell (14). In this fashion, NK cells not only play an important role in directly mediating innate effector responses but also are intrinsic to the regulation and development of adaptive immunity.

NK cells were first recognized for their ability to lyse leukaemia cells without prior activation (15) and are vital to tumour control through their ability to respond to cells with either abnormal class I major histocompatibility (MHC I) molecule expression (16) or "stress ligand" expression (17) associated with cellular transformation. NK depletion experiments have highlighted the importance of NK cells in tumour control (18, 19) and NK cells are considered crucial in the control of human and murine cancer, both through their unique ability to detect and reject malignant cells directly in the absence of inflammatory stimuli (16, 20), and through their activation of adaptive immunity (21).

Viral immunity is also an key function of NK cells, demonstrated most compellingly by the shortened lifespan due to viral disease in individuals lacking NK cells (22, 23). Additionally, both humans and mice expand specific NK cell populations in response to cytomegalovirus (CMV) (24, 25) and in response, CMV mutates in response to NK cell pressures (26, 27) and encodes proteins specifically oriented to evade NK cells (28). NK cell activation and cytokine secretion play important roles in the control of numerous other viruses, including Sendai (29), Influenza A (29), Human Immunodeficiency (30), Ebola (31) and Epstein-Barr (32). Roles are also now being described for NK cells in the control of bacterial and parasitic disease including malaria (33), tuberculosis (34), trypanosomiasis (35) and shigellosis (36).

NK cells also have a role in pregnancy, making up ~70% of lymphocytes found in the decidua. This phenotypically distinct NK cell subset appears to play a role in

controlling the invading trophoblast along with protecting the developing foetus from maternal immunity and possibly from disease (37). Pathology in pregnancy such as preeclampsia (38) and recurrent miscarriage (39) have been associated with unfavourable combinations of foetal MHC I and MHC I-dependent NK cell receptors on maternal NK cells. Non-pregnancy related pathology related to NK cells has also been described, including roles in inflammatory disease such as atopy (40), asthma (41) and autoimmunity (42) primarily through the effects of NK cell derived cytokines. For example, in the autoimmune disease multiple sclerosis, periods of remission have been associated with NK cell secretion of IL-5 while periods of active disease are associated with high levels of NK cell derived IFN $\gamma$  (12, 43).

Beyond the natural function of NK cells in pregnancy and disease, they also are of considerable importance for transplantation. The phenomenon of "hybrid resistance" where alloreactive recipient NK cells reject homozygous parental bone marrow transplanted into heterozygous F<sub>1</sub> recipients is the archetypal demonstration of the role of NK cells in transplantation biology (44). NK cells have a significant role in the failure of both allogeneic (45, 46) and xenogeneic (47, 48) bone marrow transplants primarily through the absence of MHC I alleles on the donor graft capable of being recognized by inhibitory receptors on NK cells in the recipient. Conversely, some mismatch of MHC I and inhibitory NK receptors, during haematopoietic stem cell transplantation (HSCT), has proven beneficial. The mismatch confers to NK cells in the graft the ability to eliminate unwanted malignant hematopoietic cells remaining in patients receiving therapeutic bone marrow transplant for leukaemia, a phenomena known as graft versus leukaemia (GvL). Additionally, NK cells are thought to reduce the likelihood of graft versus host disease (GvHD) and graft rejection in HSCT through slight mismatches that allow clearance of recipient DC and T cells, respectively (49).

The role of NK cells in mediating acute allogeneic organ rejection is relatively insignificant although they are found in the organ infiltrate and can be mediators of chronic rejection. They do, however, play a potent role in mediating hyperacute rejection of xenogeneic tissue as demonstrated in porcine to primate xenogeneic systems (50), primarily through the engagement of xenoreactive antibodies by NK cell Fc receptors inducing antibody dependent cellular cytotoxicity (ADCC) (51) and through NK cell



receptor interaction with carbohydrates (52) and complement bound to donor endothelial cells (53). Xenogeneic tissue also triggers NK cell motility and cytokine release (54) making NK cells important mediators of chronic rejection even after hyperacute rejection has been controlled (50). The induction of mixed chimerism, introduction of donor bone marrow into a T cell depleted host prior to tissue transplantation by the same donor, has proven to be an effective mechanism by which to facilitate both allogeneic and xenogeneic graft acceptance (55). Despite the success of mixed chimerism in eliminating the acute rejection of tissue grafts in concordant species without the use of immunosuppressive drugs, the absence of MHC I recognizable by inhibitory receptors on NK cells ultimately results in chronic rejection and loss of the tissue graft (48).

### **B. NK Cell Receptors and Their Ligands**

NK cell functions are effected through the activation of a myriad of cell surface receptors including receptors for cytokines, immunoglobulin complex binding to CD16 (FcγRIII), inducing ADCC, and through adhesion molecule binding to LFA-1 and Mac-1 (56). In addition to these receptors, NK cells also express a number of receptors for MHC I and pathogen and tumour ligands. The majority of these receptors are encoded in two specific gene complexes known as the Leukocyte Immunoglobulin-like Receptor Complex (*LRC*) and the Natural Killer Gene Complex (*NKC*) (Fig. 1-1), primarily as inhibitory and activating pairs, that act in balance to control NK activation (57, 58).

#### *Receptors of the LRC*

The *LRC* is found on chromosome 19 of humans, chromosome 7 of mice and chromosome 1 of rats (Fig. 1-1A) and encodes in each species, NK cell receptors that belong to an immunoglobulin-domain containing receptor superfamily. While this receptor complex is shared between rodents and primates it contains many more NK cell receptors in primate lineages. Despite this, there are some NK cell receptors in common, including the Nkp46 natural cytotoxicity receptor (NCR), which is found exclusively on NK cells, both in the resting and activated state (59-61). Nkp46 was originally identified for its ability to trigger lysis of both normal and transformed autologous, allogeneic and

xenogeneic cells (59, 62). Recently, hemagglutinin of Influenza virus and hemagglutinin-neuraminidase of Sendai virus have also been demonstrated as ligands for Nkp46 (63). The rodent and primate *LRC* also share in common the genes for the DAP12 and DAP10 adapter molecules that associate with activating receptors on NK cells, through charged residues found in their transmembrane domains, facilitating receptor expression and containing the signaling motifs required for activating receptor function (56). Lastly, all three species encode the leukocyte-associated immunoglobulin-like receptor-1 (*LAIR1*) gene that functions as an inhibitory receptor (64-66) and is associated with chronic Epstein-Barr virus infection in humans (67).

Although there are similarities between the rodent and primate *LRC*, with an overall syntenic gene organization, through the course of evolution primate and rodent lineages have diverged in the use of the *LRC* for encoding NK cell receptors. An ancestral *LRC* likely contained both *Nkp46* and a common ancestor to what are now the paired immunoglobulin-like receptor (*PIR*) genes in rodents and the *LAIR* and leukocyte immunoglobulin-like receptor (*LILR*) genes found in all three species (68). The *PIR* genes do not appear to function as receptors on mouse NK cells (69) but may have a member on rat NK cells (70) while only *LAIR1* and *LILRB1* (also known as *ILT2*) within the extensive *LILR* family, are expressed on NK cells in humans. *LILRB1*, also an inhibitory receptor, binds the human CMV (HCMV) MHC I "decoy" homologue, *UL18* (71) and a number of HLA class I alleles through their conserved  $\alpha 3$  domain and  $\beta 2$ -microglobulin ( $\beta 2m$ ) (72). *LILRB1* is also highly expressed on NK cells of the decidua where it interacts with HLA-G and dampens NK cell activation (73).

Additionally, the gp49A and B activating and inhibitory receptor pair are found in rodents although they are encoded outside of the *LRC* on chromosome 10 of mice (74). These receptors also belong to the immunoglobulin-domain containing receptor superfamily, are found on activated NK cells and are found to be specifically upregulated following IL-2 stimulation or murine CMV (MCMV) infection (75).

The most extensive and primary NK cell expressed gene family found in the primate *LRC* is the killer-cell immunoglobulin-like receptors (*KIR*), which are found in activating and inhibitory pairs and, unlike rodent lineages, are the predominant allele specific receptors found in primate species for classical MHC I ligands (76, 77). The

primate *KIR* gene family has undergone extensive tandem duplication and diversification events to produce marked polymorphism at both the receptor level (78) and within haplotypes, creating variability in the number, type and allelic representation of *KIR* genes within individuals (78, 79). Despite this, two main *KIR* haplotypes, A and B, can be identified (80-82) with each individual haplotype consisting of certain common genes interspersed with variable regions displaying plasticity of content (78, 79). It is thought that this marked expansion and diversification within the KIR receptor family is driven through evolutionary pressure from their highly polymorphic MHC I ligands. KIR receptors are the functional equivalent to the C-type lectin like Ly49 receptors, which are the allele specific receptors for classical MHC I in rodents and the horse (83), although they are completely different gene families (immunoglobulin-like versus lectin-like), encoded within separate NK cell gene clusters (*LRC* versus *NKC*) and share no common ancestor in evolution (84). Within mammals, KIR receptors appear to be the predominant NK cell MHC I specific receptors having been expanded in cow, cat, dog and pig (85). Despite the presence of one *KIR* gene in the rat *LRC* and two on the X-chromosome of mice, that may be functional on NK cells, KIR are not a major NK cell receptor family in rodents (86). There has been some KIR expansion in the horse but all equine *KIR* genes identified to date are non-functional (83).

In addition to the numerous immunoglobulin-like receptors encoded in the *LRC*, two additional immunoglobulin-like activating NCR receptors, NKp30 and NKp44 are worth mentioning. Both receptors are expressed in humans, and Nkp30 is found on a subset of rat NK cells (87) while mice express Nkp44 but have only a *NKp30* pseudogene. NKp30 and 44 appear to complement NKp46 in the recognition and clearance of transformed cells by NK cells (88).

#### *Receptors of the NKC*

Like the *LRC*, the *NKC* is encoded on different chromosomes in humans versus rodents, being found on human chromosome 12, mouse chromosome 6 (56) and rat chromosome 4 (89). (Fig. 1-1B) Unlike the *LRC*, both primate and rodent species encode a number of common NK cell receptors within the *NKC*. The NK cell receptors encoded in the *NKC* are all members of the C-type lectin receptor superfamily (90, 91), are type II



transmembrane proteins and are expressed as disulfide linked dimers on NK cells (92-94). Like receptors found in the *LRC*, *NKC* receptors are commonly found as inhibitory and activating pairs, with inhibitory signals typically dominating over activating signals, in balancing NK cell responses to potential targets (57, 58, 95).

One of the first *NKC* receptor types to be identified was the activator NKR-P1 on rat NK cells (94, 96). Numerous Nkrp1 receptors are found in mice. Some Nkrp1 gene products react with the PK136 antibody and are equivalent to the commonly used NK cell marker, NK1.1. (97). Nkrp1c, Nkrp1a and f have been identified as activating receptors whereas Nkrp1d and b are inhibitory receptors (98, 99) variably expressed on NK cells and NKT cells (100) between inbred strains of mice. Ligands for some Nkrp1 have been identified as C-type lectin related (Clr) molecules, which are also encoded in the *NKC* and are expressed on NK cells, DC, macrophages and osteoclasts (101). Since Clr are functional receptors themselves, engagement of Nkrp1 receptors with Clr may not only regulate NK cell function but also the function of the cell expressing the Clr ligand (102). In contrast, only the NKR-P1A receptor, which interacts with the Clr-like, lectin-like transcript-1 (LLT1) on target cells (103) and is also encoded in the *NKC*, has been identified in human and is expressed on subsets of NK and T cells (104).

The NKG2 family was first identified in human (105) as heterodimeric receptors coupled with CD94 (106). The NKG2A/CD94 receptor acts as an inhibitory receptor (107), whereas the NKG2C/CD94 receptor is activating (108). Found in both human and mouse, these receptors recognize the non-classical MHC I ligands HLA-E and Qa-1<sup>b</sup>, respectively, which present peptides derived from the leader sequences of classical MHC I, presumably allowing NK cells to survey for the global expression of classical MHC I. Like other NK cell receptor pairs, the inhibitory NKG2 molecule signaling dominates over activating molecule signaling on the same NK cell, thereby preserving tolerance (109).

The NKG2D receptor, while sharing similar nomenclature, is not closely related to other NKG2 molecules and is expressed as an activating homodimer on NK cells, NKT cells,  $\gamma\delta$  T cells, activated CD8<sup>+</sup> T cells and macrophages (17, 110, 111). It primarily recognizes ligands upregulated through activation of the DNA damage response pathway, which commonly occurs in infected or transformed cells (112), including in



humans, MHC class I chain related proteins A and B (MIC-A and MIC-B) and UL16 binding proteins (ULBPs) (17, 113) and in mice, the minor histocompatibility molecule, H-60, members of the retinoic acid early transcript-1 family (Rae-1) (110, 111) and the ULBP-like molecule, Mult-1 (114). All the ligands for NKG2D are MHC I-like and appear to be inducible under conditions of "stress" or pathology (115, 116). Both HCMV and MCMV are known to encode viral proteins capable of interfering with NKG2D ligands (113, 117).

Additionally, numerous less well described NK cell receptors have been identified in the *NKC*, including the killer cell lectin-like receptor subfamily F1 and G1 receptors (KLRF1, KLRG1), LST1/Clr, activation induced C-type lectin (AICL), and CD69 (118).

Lastly, the *NKC* of rodents and the horse encode the *Ly49* gene family, which, like the *KIR* gene family in primates, serves as the predominant allele specific receptors for MHC I. Since *Ly49* receptor genes exist as only a single copy in cat, dog, pig and in primate species including humans (85, 119), it appears that only rodents and the horse have expanded the *Ly49* gene cluster. This *Ly49* expansion has occurred through a complex series of duplications, deletions and conversion events, remarkably similar to but completely independent of the genetic expansion of *KIR* receptor genes in primates, to maintain receptors capable of interacting with their highly polymorphic classical MHC I ligands (120-122) in what appears to be a remarkable display of convergent evolution between species. It is this highly polymorphic and polygenic *Ly49* receptor family within rats and mice, and their allele specific interaction with classical MHC I, that form the major focus for this body of work.

### **C. The Murine *Ly49* Receptor Repertoire**

*Ly49* receptors are encoded in the *NKC* of mice and rats and appear to have arisen from a common ancestral *Ly49* cluster (123), although so much expansion and diversification of the cluster has occurred since speciation ~20 million years ago, that only a single ortholog, *Ly49B* in mice and *Ly49i13* in rat, can be identified between these two species (124). Both rat and mouse *Ly49* are type II transmembrane proteins (58, 125) expressed at the cell surface as disulfide bonded homodimers and each 40-45kD subunit is made up of a cytoplasmic, transmembrane, stalk and C-type lectin-like domain (CTLD)

(92, 126, 127) (Fig. 1-2). The Ly49 CTLD is similar to the carbohydrate recognition domain (CRD) of C-type lectin receptors and can bind carbohydrate, but Ly49 do not conserve the residues required for calcium binding found in typical C-type lectins and typically interact with MHC I in a carbohydrate independent manner (128). Activating and inhibitory forms of Ly49 exist in both rat and mouse and, like KIR2DL4 identified in humans (129), rat also contains bifunctional Ly49 receptors capable of both activating and inhibitory function (124). Bifunctional receptors are absent in the mouse strains examined to date (120, 121, 130).

Activating and inhibitory Ly49 share sequence homology in their extracellular stalk and CTLD domains, with many highly homologous activating/inhibitory receptor pairs being identified in both species (131, 132). Inhibitors differ from activating receptors by the presence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) (125) in their cytoplasmic tail that becomes tyrosine phosphorylated upon receptor ligation. The phosphorylated ITIM then recruits src homology 2 domain-containing tyrosine phosphatase – 1 or 2 (SHP-1 or SHP-2) (133) or SH2 domain containing inositol-5 phosphatase (SHIP) (134) resulting in the desphosphorylation of substrates required for NK cell activation, thereby dampening or eliminating NK effector responses. Which phosphatase is recruited may depend on the activation pathways being modulated (135) (Fig. 1-2). In contrast, activating Ly49 do not contain an ITIM motif in their cytoplasmic domain but instead, contain an arginine residue in their transmembrane domain facilitating association with the ~12kD signaling adapter protein DAP12 through an aspartic acid residue within its transmembrane domain (136). The DAP12 signaling adapter is also a disulfide bonded homodimer and contains immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic domain of each subunit (136). The DAP12 adapter is required both for activating Ly49 receptor function and for optimal expression. Upon receptor ligation, it is the ITAM motifs in the DAP12 homodimer that become phosphorylated on tyrosine, recruiting syk tyrosine kinase and inducing phosphorylation of key substrates such as phospholipase C and c-Cbl, ultimately leading to the activation of the MAPK cascade and NK cell activation (137) (Fig. 1-2)

Numerous activating and inhibitory Ly49 receptors have been identified in the rat and mouse. More than 20 Ly49 genes have been described in mouse, 13 of which are

inhibitory (*Ly49a, b, c, e, f, g, i, j, o, q, s, t, v*) and eight that are activating (*Ly49d, h, l, m, p, r, u, w*) (136). Even more *Ly49* have been identified in rat, 34 to date, including nine pseudogenes, 13 inhibitory (*Ly49i1 – i13*), eight activating (*Ly49s1 – s8*) and five bifunctional receptors (*Ly49si1 – si5*). The bifunctional receptors contain both an ITIM and a charged transmembrane domain for interaction with DAP12 (124). Not all *Ly49* genes are present within the *NKC* of different rat and mouse strains. The *NKC* of the C57BL/6 mouse strain contains 16 *Ly49* genes whereas the BALB/c strain contains only nine, and 19 genes are found in the *NKC* of 129/Sv mice (120, 121, 130). Although all three strains similarly encode *Ly49q, e, i, g, c* and *a* in what appears to be 'framework' positions of the *NKC*, similar to the framework *KIR3DL3, 2DL4* and *3DL2* genes found in the human *LRC*, each framework region is interspersed with a variable number and type of *Ly49*. These variable *Ly49* haplotypes found between strains can also contain different strain specific alleles of similar genes (123).

In the rat, only the BN strain's *NKC* has been fully sequenced (138) and like known mouse *NKC* haplotypes, contains a number of both functional and non-functional *Ly49*. A non-functional *Ly49* gene found in one strain may be found as a functional transcript in another and these *Ly49*, along with other *Ly49* pseudogenes, appear to be both the casualties of and the breeding grounds for the extremely rapid evolution of this receptor family in both species (123). Despite the absence of more than one fully sequenced rat *NKC*, there is strong evidence that inbred rat strains also differ in their *NKC* haplotypes, evidence that originated in studies of allogeneic lymphocyte cytotoxicity (ALC), which is mediated by NK cells (139), and has been confirmed by restriction fragment length polymorphism analysis (140) and amplification of *Ly49* receptors from different rat strains (124). Interestingly, PVG strain rats that are known to be strong NK cell alloresponders are now also known to have haplotypes that contain a large number of activating *Ly49* genes, whereas low alloresponding strain haplotypes contain few or no activating *Ly49*. We now know that the originally defined *Nka* locus, that mapped to the distal portion of the *NKC* and defined rat NK cell alloreactivity (141), encodes all of the purely activating *Ly49* found in rat (124).

This extensive diversity between species and strains appears to have arisen from the need of NK cell receptors, which cannot rearrange like the receptors of adaptive



immunity, to "keep up" with the highly diverse MHC I alleles that act as their ligands and possibly also in response to MHC I homologues encoded by viruses (124). Phylogenetic examination of Ly49 in rat and mouse indicates that rapid expansion and diversification has occurred in both species through multiple duplication, homogenization by gene conversion and deletion events (89, 121, 124). It is also evident that activating Ly49 appeared after the evolution of inhibitory Ly49, most of which derive from a common activating domain that may have appeared after Murinae separated from other rodent lineages ~31-34 million years ago. The bifunctional receptors found in rat, along with the rat Ly49s3 receptor, segregate in an independent cluster indicative of a possible second emergence of an activating domain in this species and gives credence to the hypothesis that inhibitors first obtain an activating domain and then later lose their cytoplasmic ITIM motifs (142).

Expression of activating and inhibitory Ly49 receptors is heterogeneous and results in subsets of NK cells expressing partially overlapping repertoires of usually one to six of the Ly49 from the given *NKC* haplotype. Ly49 expression, for the most part (143), appears to be stochastic and independent (144, 145) even for alleles (146) although the expression of one inhibitory receptor capable of recognizing an autologous MHC I allele can down regulate or prevent the expression of a second inhibitor with similar allelic specificity for MHC I. Variegated expression of Ly49 is controlled by the presence of a bidirectional promoter that acts as a "probabilistic switch" in immature NK cells determining whether a *Ly49* gene will be turned "on" or "off" in mature NK cells (147, 148). Since inhibitory Ly49 receptor interaction with self MHC I appears to affect the expression of other Ly49 (149, 150), the sequential expression of Ly49 from probabilistic promoters may occur until NK cells express at least one inhibitory Ly49 capable of recognizing a self MHC I allele, thereby maintaining tolerance to self (149-153).

#### **D. Class I MHC**

Major histocompatibility molecules (MHC) exist in all vertebrates, with the exception of agnathans (jawless fish) (154), and are co-dominantly expressed on virtually all nucleated cells in the body. The *MHC* complex is encoded on chromosome 20 in the rat as the *RT1* complex, is represented by the mouse *H-2* complex on chromosome 17 and



by the *HLA* complex on chromosome 6 of humans (155) (Fig. 1-3). All three species contain *MHC I*, *MHC II* and *MHC III* genes within their respective *MHC* complexes. Mouse and rat have an orthologous arrangement of genes at this locus starting with a centromeric region of *MHC I*, followed by a region containing *MHC II* genes, a region containing *MHC III* genes and finally a large telomeric region of numerous additional *MHC I* genes. The human complex is similar with the exception of the centromeric *MHC I* region (155). Although genes within all three regions play important roles in mediating immune responses it is solely the *MHC I* gene products that serve as ligands for NK cell receptors.

*MHC I* molecules can be classified into two major subgroups, classical Ia molecules (*MHC Ia*) and non-classical Ib molecules (*MHC Ib*). *MHC Ia* are highly polymorphic and important for antigen presentation to and recognition by CD8<sup>+</sup> T cells (156). They include *H-2K* centromeric to the *MHC II* genes and *H-2D* and *L* telomeric to the *MHC III* genes in the mouse and *RT1-A* genes found only centromeric to the *MHC II* genes in the rat (155). The *MHC Ia* loci within the *MHC* complex each code for an *MHC I* heavy chain which non-covalently associates with the small globular and relatively invariant  $\beta$ 2-microglobulin ( $\beta$ 2m) molecule, an association that is required both for proper folding of the heavy chain and expression at the cell surface. The *MHC I* heavy chain consists of 3 domains,  $\alpha$ 1,  $\alpha$ 2 and  $\alpha$ 3. The  $\alpha$ 1 and  $\alpha$ 2 domains each consist of four anti-parallel  $\beta$ -strands followed by one long  $\alpha$ -helix. These domains associate to form a  $\beta$ -sheet platform bordered by parallel  $\alpha$ -helices creating a groove within the *MHC I* that can bind short peptides for presentation to CD8<sup>+</sup> T cells. The  $\alpha$ 3 domain is membrane proximal to the peptide-binding groove, and along with residues below the peptide-binding platform, forms contacts with  $\beta$ 2m (157) (Fig. 1-4)

*MHC Ib* also consist of a heavy chain encoded in the *MHC* complex. Some of these molecules associate with  $\beta$ 2m while others do not, and only a few non-classical molecules have been shown to bind peptide (155). The *MHC Ib* regions in rat and mouse are large and diverse and genes encoding *MHC Ib* are found in clusters interspersed between regions of non-*MHC I* framework genes in the telomeric *MHC I* region of both species (142). In contrast to the ubiquitously expressed and highly polymorphic *MHC Ia*, *MHC Ib* molecules tend to be monomorphic or oligomeric, have more restricted

expression profiles and tend to have specialized functions in immunity. Although rat does not contain *MHC Ia* genes in its telomeric *MHC* region like mice, the most proximally encoded *MHC I* genes in the rat *MHC Ib* complex are Ia-like (158, 159). At least one rat *MHC Ib* allele has been shown to present peptide to CD8<sup>+</sup> T cells (160) and the *RT1-U* *Ib* locus has been shown to mediate cytotoxic T lymphocyte (CTL) responses to alloantigens (161). Despite this, these *MHC Ia*-like genes are not orthologous to mouse *H-2D/L* and are not major loci for CTL responses compared to *H-2K/D/L* and *RT1-A*.

Mouse and rat do share a number of *MHC Ib* orthologs including *H2-T* like genes in the second *Ib* cluster (162), and the *H2-M* like genes in the third and fourth *Ib* clusters (162, 163). For example, rat RT.BM1, found in the second *Ib* cluster is directly orthologous not only to mouse Qa-1<sup>b</sup> but also to human HLA-E and similarly presents peptides derived from the leader sequences of *MHC Ia* molecules (164). Despite the presence of some *MHC Ib* orthologs between mouse and rat, and even human, there are no orthologs present among the highly polymorphic *MHC Ia* molecules. This is surprising considering the close evolutionary relationship between rats and mice, and was under debate for some time regarding the *MHC Ia* genes encoded centromerically to the *MHC II* region, primarily because these genes appear to have derived via a translocation event from the telomeric *MHC I* region into identical insertion points (155). Regardless, due to the rapid evolution of the *MHC I* genes in rats and mice, involving multiple non-synonymous mutations, amplification and deletion events (165-168), no clear ortholog is seen between rat and mouse *MHC Ia* alleles. As an example of this extensive diversification, only one *MHC Ia* gene, *H-2K*, is encoded in the centromeric region of mouse, while between one and three *RT1-A* genes can be encoded in this region in rat, depending on the inbred strain (158, 169). Similarly, in inbred mouse strains, not all strains encode *H-2L* in the telomeric *MHC Ia* locus (170).

This strain difference at the *RT1-A* and *H-2L* loci highlights the diversity of rat and mouse *MHC Ia* genes; not only a diversity in gene number but also of allelic forms of genes resulting in different *MHC* haplotypes in inbred strains. Because *MHC Ia* are important for the binding and presentation of peptides to CTL in mediating adaptive immune responses, it is understandable that a great deal of polymorphism would be generated in the peptide binding groove of *MHC Ia* molecules, facilitating the binding of

the greatest diversity of peptide fragments to ensure that immunodominant peptides capable of eliciting productive responses by CTL can be presented. This has resulted in a large number of *MHC Ia* alleles in mouse and rat (171). The overall *MHC* gene content found within inbred rat and mouse strains therefore, have each been assigned an allelic designation that denotes the gene number, type and allelic variations of each strain's *MHC* complex. For example, the BN strain rat contains three *RTI-A* genes, a variable number of *MHC Ib* genes and polymorphic versions of each that have collectively been denoted as the RT1<sup>n</sup> haplotype. Conversely, the RT1<sup>c</sup> haplotype contains two *RTI-A* genes, again variable *MHC Ib* gene content and polymorphic versions of each that differ from the BN strain. Similarly, haplotypes such as H-2<sup>b</sup> in the C57BL/6 mouse distinguishes its MHC I allele products from those found in the BALB/c mouse, which has a H-2<sup>d</sup> haplotype. Non-*MHC I* genes can also have allelic variances, in particular the *MHC II* genes which present peptides to CD4<sup>+</sup> T cells, and notably in the case of the rat, the transporter for antigen presentation (*TAP*) genes, which exist as both *TAP-A* and *TAP-B* alleles, and function differentially in rat strains in their ability to transport peptides into the endoplasmic reticulum for loading into newly formed MHC I (172).

The peptide binding properties of MHC I molecules has allowed further classification of MHC I alleles into what are known as supertypes. Supertypes were first identified in human vaccine studies that aimed at identifying peptides motifs that could be bound by a wide range of HLA MHC I alleles. MHC I molecules do not rely equally on every peptide residue to bind it into the groove, instead forming strong associations with two or three anchor residues in the peptide, binding them deeply into anchor binding pockets. Typically, MHC I bind the second (P2) and last (P9) residues of peptide although P4 and P5 residues can act as anchors in some alleles. In the examination of peptide binding preferences of hundreds of HLA alleles, and now of other primate, mouse and rat MHC I alleles (173) it appears that MHC I from each species can be categorized into about nine or 10 different supertypes based on their anchor binding preferences. The presence of identical supertypes in species that do not share any MHC Ia orthologs precludes that supertype motifs have been conserved from a common ancestor but are instead likely a result of convergent evolution. Why MHC I alleles from divergent species have all evolved to have about 10 different binding specificities may be



due to advantages to MHC I stability (174), pressure to maintain MHC I molecules capable of binding peptides generated by peptide loading machinery (175) and/or pressure to present immunodominant peptides in a context that is recognizable by MHC restricted T cells. The overriding pressure and importance to maintain MHC I molecules that are recognizable by T cells and capable of binding an enormous repertoire of peptide antigens may also be the driving force behind the evolution of the extremely large and diverse innate repertoires of MHC I allele specific NK cell receptors, particularly KIR in human and Ly49 in rat and mouse, capable of recognizing cells deficient in MHC I expression.

#### **E. Allele Specific Ly49 Recognition of Class I MHC**

MHC I present both self and non-self peptides on the surface of body cells. The presentation of self-peptides is important in the education of CD8<sup>+</sup> T cells to remain self-tolerant while the presentation of non-self peptides, from intracellular viruses and parasites, induces T cell activation and CTL activity. Tumour cells, while derived from self, can also present an altered peptide repertoire recognized as non-self by CD8<sup>+</sup> T cells due to altered biosynthesis and over or inappropriate expression of proteins in ontogeny, for example, expression of foetal genes in an adult malignancy. In an attempt to evade recognition by CTL, pathogens have evolved means to disrupt normal assembly and transport of MHC I while tumorigenic cells often evade CTL detection by dysregulation of gene expression resulting in loss of MHC I (77). This loss of MHC I can be total or allele specific (176, 177). The expression of receptors for MHC I allows NK cells of innate immunity to recognize and eliminate these cells with altered MHC I expression. While the NKG2/CD94 family plays a partial role in this surveillance through the recognition of global MHC I loss, allele specific Ly49 receptors can detect not only the total loss of MHC I but also the selective down regulation of particular MHC I alleles.

Both the activating and inhibitory Ly49 expressed on NK cells are MHC I allele specific. Of the rodent Ly49 characterized to date, many have only one or a few MHC I alleles that they can recognize. For example in the mouse, the inhibitory Ly49G has been demonstrated to recognize H-2D<sup>d</sup>, L<sup>d</sup> and D<sup>k</sup> and the inhibitory Ly49A has been shown to recognize H-2D<sup>d</sup>, D<sup>p</sup> and D<sup>k</sup> (178) while in the rat, Ly49i2 has been shown to recognize



RT1-A1<sup>c</sup> (179). Other inhibitory Ly49 exhibit a broader range of recognition, for example, mouse Ly49C, which can recognize H-2K<sup>b</sup>, K<sup>d</sup>, K<sup>k</sup>, D<sup>d</sup> and D<sup>b</sup> (178). Allelic differences in the same receptor can also affect MHC I allele specificity, for example, the BALB/c allele of Ly49G can recognize both H-2D<sup>d</sup> and D<sup>k</sup>, whereas the C57BL/6 allele recognizes only H-2D<sup>d</sup> (180). Under normal circumstances, the signals from these inhibitory Ly49, upon recognition of MHC I alleles on potential target cells, dominate over the activating signals from virtually all other receptors on NK cells (123, 181, 182). Therefore, NK cells maintain tolerance to cells that express normal levels of at least one MHC I allele that can be recognized by an inhibitory Ly49 receptor on the NK cell surface. Upon the down regulation of self MHC I by pathogenically altered cells, this dominant inhibition is released, triggering target cell elimination through activating Ly49 and other activating receptors on the cell surface. This phenomenon was first described by Karre's group and is known as the "missing self hypothesis" (183). The allele specificity demonstrated by Ly49 for MHC I products is particularly advantageous in that it allows for the detection of the selective down regulation of specific allele products. This would, for example, prevent the escape of viruses that selectively down regulate an MHC I allele that is particularly effective at presenting viral peptide for CTL recognition and clearance, but leave normal expression of other MHC I alleles at the cell surface for NK cell recognition (184).

Activating Ly49 receptors can also recognize MHC I and show allele specificity, recognizing only one or a few MHC I alleles (178). For example, mouse Ly49P<sup>NOD</sup> recognizes H-2D<sup>d</sup> while Ly49W recognizes H-2D<sup>d</sup> and D<sup>k</sup> (185, 186). No conclusive ligand has yet been demonstrated for a rat activating Ly49 but strong evidence exists to indicate that rat activating Ly49 recognize specific alleles expressed from the *MHC-Ia* like, *MHC Ib* locus. For example, BN strain NK allorecognition of WF targets is mediated by the *RT1-E<sup>n</sup>* locus, and like the dominating effects of inhibitory Ly49 in mouse, could be inhibited by the co-expression of RT1-A1<sup>n</sup> on RT1-E<sup>n</sup> expressing cells (187). While not conclusively mediated by a specific Ly49 in the rat, these effects do map to the NKC and along with extensive ALC work implicating rat Ly49 in the allele specific recognition of MHC I (139, 140, 188), indicates that rat Ly49 systems are subject to the same allelic specificity as mouse systems.

Mouse and rat Ly49 also appear to recognize xenogeneic MHC I in an allele specific manner, a phenomenon first recognized in the production of xenogeneic bone marrow chimeras for transplantation (189-191). Xenogeneic recognition has primarily been examined for the mouse activating receptor Ly49D. First identified as the *CHOK* locus capable of mediating cytotoxicity against Chinese hamster ovary cells (CHO), it is now known that *CHOK* encodes Ly49D (192) which specifically recognizes the hamster Hm1-C4 allele of MHC I (193). Ly49A and G additionally recognize a currently unidentified ligand within CHO cells (194). Furthermore, Ly49D recognizes MHC I targets specifically of the rat RT1<sup>lv</sup> and RT1<sup>l</sup> MHC I haplotypes and not other haplotypes (195). We too have shown that the BALB/c and C57BL/6 allele products of Ly49G differentially recognize rat cells from different MHC I haplotypes, with the BALB/c allele recognizing RT1<sup>c</sup> and RT1<sup>l</sup> expressing targets and the B6 allele recognizing only RT1<sup>c</sup> targets (180).

How and why a system of paired activating and inhibitory receptors exists in rodents for the allele specific recognition of MHC I has been an issue of considerable debate. Particularly since many activating and inhibitory Ly49 pairs share extremely high homology in their CTLD (132) and recognize identical MHC I allelic repertoires. For example, Ly49G<sup>BALB/c</sup> and W, that have 97.6% identity in their CTLD both recognize H-2D<sup>d</sup> and D<sup>k</sup> (185). It is comprehensible that since inhibitory Ly49 dominate over activating Ly49 on the same NK cell, that if the MHC I allele recognized by the inhibitory receptor was lost and an MHC I allele recognized by the activator remained, that NK cell activation would ensue. In reality, the Ly49 expression patterns seen on NK cells would indicate that for every activator expressed there is at least one inhibitor with an overlapping ligand repertoire capable of modulating its activity (196) and within inbred strains typically only allo-reactive activating Ly49 are found (120, 121, 130, 178, 185, 186), bringing into question how often such a scenario would occur. Upon closer inspection, this system of paired activators and inhibitors appears to be finely tuned to alterations in ligand density in the context of paracrine indicators of immune activation (181, 197). For example, H-2D<sup>d</sup> induced Ly49D activation can overcome Ly49G inhibition through the same ligand in the presence of IL-12 or 18, which appear to act as a "second signal" much like is required for T cell activation (181). Activators may also

play a crucial role in the recognition of viral MHC I homologues, which could otherwise act as "decoys" for inhibitory Ly49 by viruses that down regulate MHC I. For example, Ly49I from the MCMV susceptible 129/Sv strain of mice recognizes the MCMV MHC I homologue m157, whereas the Ly49I allele from resistant C57BL/6 mice does not (198). Additionally, MCMV resistant C57BL/6 mice encode the Ly49H activating receptor, which also recognizes m157 (198), triggering clearance of infected cells and vigorous IFN $\gamma$  secretion promoting effective adaptive responses (198-201). It is suggested that activating Ly49, which make up a large percentage of the non-functional genes in the NKC, may undergo a particularly rapid birth and death rate in that they would be selected in the presence of a viral threat but deselected due to their predilection for auto-MHC I responses once such a threat has passed (202). Currently, it is under debate whether activating Ly49 evolved specifically for their role in detecting subtle variations in allelic MHC I expression or whether they first appeared through the introduction of an activating domain into an inhibitory Ly49 capable of recognizing a viral homologue, thereby maintaining residual cross reactivity for self MHC I alleles and resulting in the highly homologous activating and inhibitory pairs found within inbred mouse strains (202).

Regardless, the allele specific nature of Ly49 recognition allows far greater immune fitness in situations where only a particular subset of MHC I alleles are down regulated as well as providing a way for immediate recognition of pathology without the need for de novo synthesis of recognition receptors or ligands. The unlinked mingling of diverse Ly49 receptor haplotypes, expressed stochastically and independently, with diverse MHC I haplotypes may also provide a means by which activating Ly49 specific for viral MHC I homologues could be expressed with reduced threat of self-reactivity and autoaggression.

#### **F. Background and Rationale**

A great deal of work has been done to try to explain the allele specific molecular interaction between mouse Ly49 and their MHC I ligands while nothing is known of the molecular interaction between rat Ly49 and MHC I. Very early mouse work isolated MHC I recognition to the CTLD of Ly49 (203) and showed that Ly49 recognition of



MHC I was peptide dependent but not selective (204). This was followed by work implicating the  $\alpha 1\alpha 2$  domains through antibody blocking assays (204-206) and domain substitution experiments (128, 207, 208). Since recognition appeared to be mapping to the residues defining the peptide binding groove, others made mutations within the groove that, in combination, could disrupt recognition (209). This reintroduced the possibility that like KIR, some Ly49 receptors could be influenced by the bound peptide, something that Franksson et al. confirmed by loading H-2K<sup>b</sup> with different peptides with equal capacity to stabilize K<sup>b</sup> but with drastically different capacities to trigger inhibition through Ly49C (210). Other groups focussed on the role of carbohydrates in mediating recognition (128, 211) and identified residues in Ly49 that altered their ability to recognize panels of MHC I ligands (212).

All of this work made respectable headway into describing the Ly49/MHC I interface and much of it was confirmed upon solving of the first Ly49/MHC I co-crystal structure of Ly49A bound to H-2D<sup>d</sup> (213). This crystal structure while answering some questions, also introduced a new question since the Ly49A dimer was found to bind at two different sites on H-2D<sup>d</sup> and it was unclear which, or if both sites, were biologically relevant (Fig. 1-4A). Site 1 was located at the end of the peptide-binding groove near the N-terminal portion of the peptide, was only about 1000Å<sup>2</sup> but had exquisite ionic complementarity. This site was initially favoured as the biologically relevant site for interaction with Ly49 as it was highly polymorphic and thereby capable of mediating allele specificity, involved the carbohydrate attachment site shown previously to influence recognition (211) and was the most plausible site explaining the loss of recognition upon mutagenesis of the  $\alpha 1$  and  $\alpha 2$  domains.

In contrast, site 2 was considered a possible artefact of crystallization. The site was quite large, 3400Å<sup>2</sup>, had poor shape complementarity, encompassed a highly conserved area involving solvent exposed residues below the peptide-binding groove, in the  $\alpha 3$  domain and on  $\beta 2m$  and was bound primarily through polar bonds and H<sub>2</sub>O molecules (214). Additionally, it was considered improbable that such a conserved site could mediate allele specific interactions with Ly49. Therefore, work ensued to establish which site, 1 or 2, or both, were biologically relevant.



Additional mutagenesis in the  $\alpha 1\alpha 2$  domains, including the peptide binding groove appeared to support site 1 for Ly49A and D recognition of H-2D<sup>d</sup> (215) and it was not until 2001 that Matsumoto et al. (216) demonstrated unequivocally that site 2 was the relevant interaction site, involving residues in all three domains of the H-2D<sup>d</sup> heavy chain and involved  $\beta 2m$ . Other groups proceeded to describe more fully the interaction required with  $\beta 2m$  (217-219) and the polymorphisms found in the Ly49 that influenced specificity for MHC I (219-222). Upon the crystallization of Ly49C with H-2K<sup>b</sup> (223), showing a single binding mode at site 2 (Fig. 1-4B), it became accepted that site 2 was the biologically relevant site for interaction with MHC I.

The question as to how different Ly49 exhibit such diverse allele specificity for a ligand at a site which is virtually identical in all rodent MHC I is still an issue of considerable debate. Work by Matsumoto (224) and Nakamura (225) et al. continued to hint at a mechanism related to the conformations of the  $\alpha 1\alpha 2$  domains but no concrete model explaining allele specificity at this conserved site has been proposed. Although differential modes of dimerization, seen in crystal structures of Ly49C (223) and Ly49I (226) compared to Ly49A (213) was once considered as an explanation for the wide range of MHC alleles recognized by these receptors compared to the more restricted Ly49A, the recent crystallization of Ly49A in a dimerization state similar to Ly49C and I (227) has now drawn question to this hypothesis.

Despite all of this work, the molecular basis of mouse Ly49 allele specificity for MHC I remains unsolved. Additionally, there is an ever-growing repertoire of allele specific Ly49 being found in the rat (89, 124, 228). This repertoire has evolved independently of the mouse Ly49 repertoire and shows allele specificity for MHC I ligands that have also significantly diverged from mouse MHC I, bringing into question whether rat Ly49 also interact with MHC I at site 2 and what the molecular basis of allele specificity is in this species.

Since xenogeneic recognition had previously been demonstrated between mouse Ly49 and rat cells expressing specific MHC I haplotypes (195), including some of my own work (180), we considered that by identifying the MHC I ligands for these xenoreactive Ly49 it may be possible to identify a recognition motif shared between the two species that mediates allele specific recognition. The considerable divergence of rat

and mouse MHC I being an aid in making this conserved recognition motif on MHC I 'stand out' against otherwise divergent species-specific motifs unrelated to Ly49 recognition. Chapter I describes the identification of such a rat xenoligand for mouse Ly49 receptors, which along with being a relevant finding for rat/mouse mixed chimerism experiments, allowed us to propose a hypothesis describing what we believed to be the shared motif mediating both mouse xenorecognition and rat syngeneic recognition of a rat MHC I molecule. Chapter II then investigates this hypothesis that Ly49 receptors distinguish between MHC I alleles based on polymorphisms specifically found in the supertype-defining and anchor-binding pockets of MHC I. Lastly, chapter III, for the first time, describes in detail the molecular interaction between a rat Ly49 receptor and its MHC I ligand and maps out the variable usage of conserved residues on MHC I for both a new mouse and a rat Ly49/MHC I allele combination compared to the known Ly49A/H-2D<sup>d</sup> and Ly49C/H-2K<sup>b</sup> interactions. By comparing the variable usage of residues on MHC I by different Ly49/MHC I allelic pairs, in the context of our findings that the conformation of the polymorphic anchor binding pocket(s) affect recognition, we propose a comprehensive model of how Ly49 receptors may mediate allele specific recognition at a conserved interaction site of MHC I as defined by conformational differences in the  $\alpha 1\alpha 2$  domains.

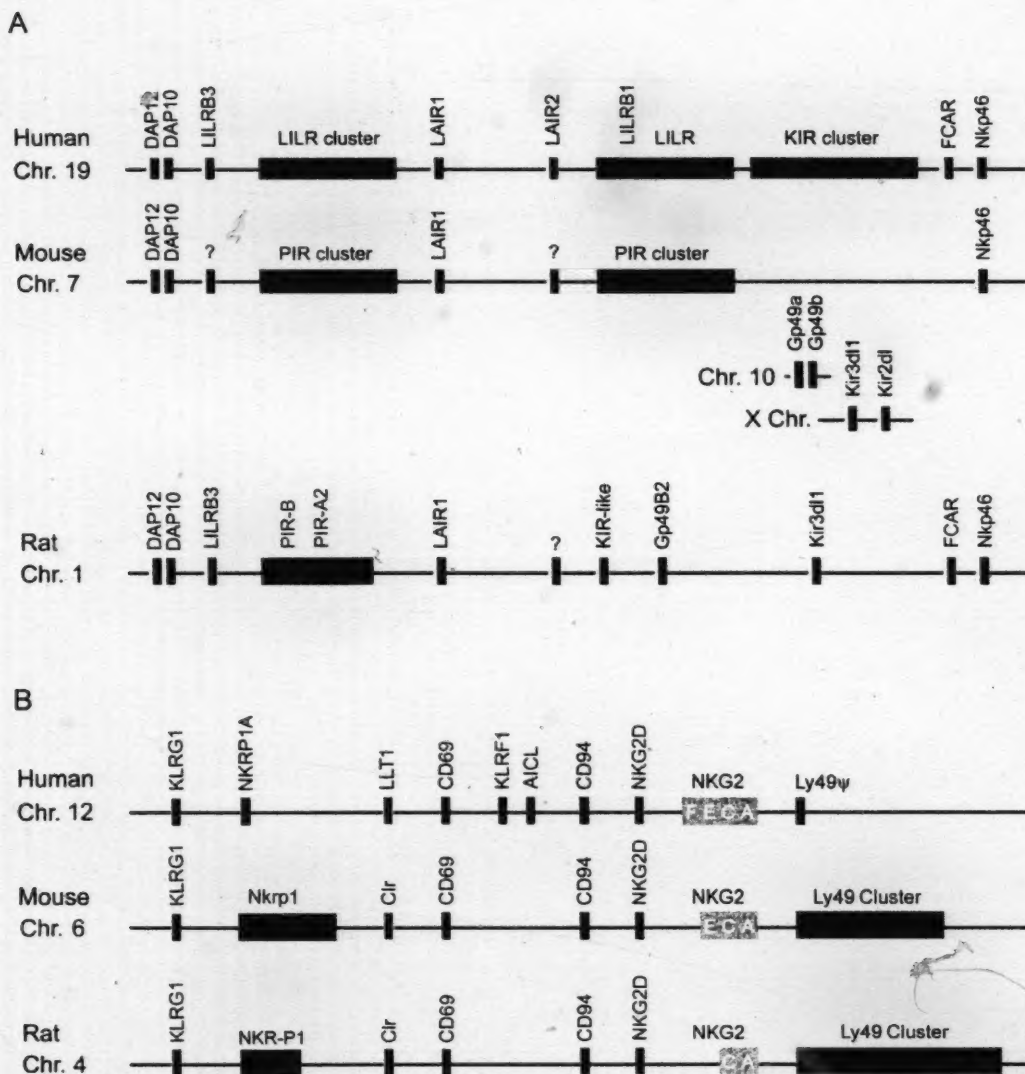


**FIGURE 1-1.** NK cell receptor genes in the LRC and NKC of human, mouse and rat. **A**, Comparative LRC gene arrangement of human, mouse and rat. Signalling adapter molecules used by NK cell receptors are in green, receptors known to be expressed on NK cells in blue and red and the functional homologues to Ly49 receptors, KIR, in red. **B**, Comparative NKC gene arrangement of human, mouse and rat. All genes shown are expressed on NK cells with the exception of the single *Ly49ψ* in humans. Light and dark green genes are known to act as receptor/ligand pairs, light and dark blue genes are expressed as heterodimers. Variably sized *Ly49* gene clusters are shown in red. Although numerous *Clr* genes exist in the mouse and are intermingled with *Nkrp1* genes, a single locus is shown for simplicity and to represent the synteny found between species.

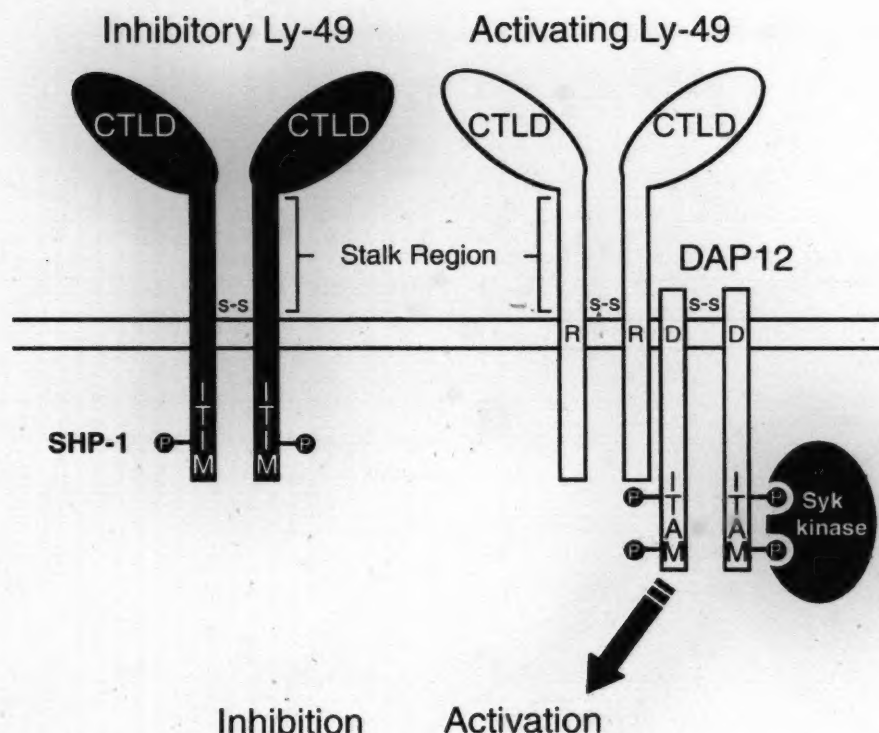


**FIGURE 1-2.** Activating and inhibitory Ly49 structure and signaling pathways. Both kinds of Ly49 are type II, disulphide bonded homodimers with similar structure in the stalk and CTLD. Upon ligand engagement, inhibitory Ly-49 receptors become phosphorylated on tyrosine within ITIM motifs in the cytoplasmic tail of the receptor and recruits phosphatases such as SHP-1, leading to suppression of NK-cell activation. In contrast, activating Ly-49 receptors associate with the signaling adapter protein DAP12/KARAP through charged residues in their transmembrane domains, arginine (R) and aspartic acid (D), respectively. As a consequence of ligand binding by activating Ly-49 receptors, the associated DAP12/KARAP becomes phosphorylated on tyrosines within ITAM motifs and recruits Syk kinase, leading to NK cell activation. Adapted from Kane K. P., Lavender K. J. and B. J. Ma. 2004. Ly-49 receptors and their functions. *Crit. Rev. Immunol.* 24(5): 321-348.



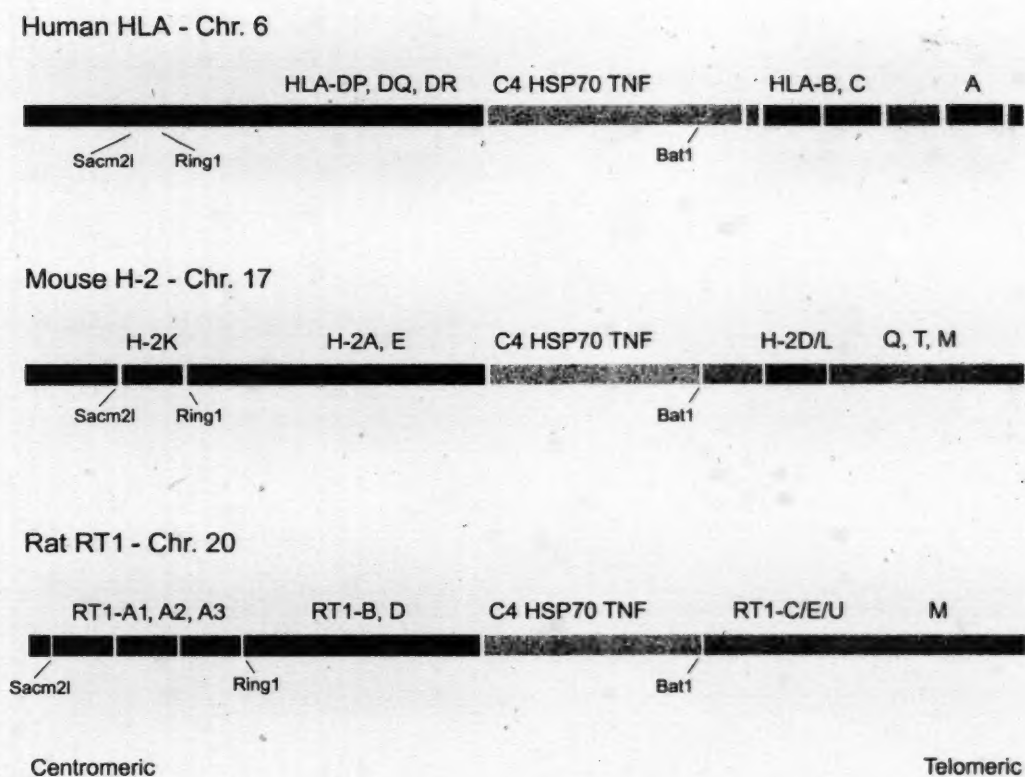


**FIGURE 1-3.** Comparison of the human, mouse and rat MHC Complexes. Genes are shown in the order they are found on the chromosome from centromere to telomere. The classical class Ia regions are in red, class II region in blue, class III region in green and non-classical class Ib region in orange. Representative genes from each region are listed above the chromosome. Framework genes marking some of the orthologous MHC I boundaries between species are listed in lowercase below the chromosome.

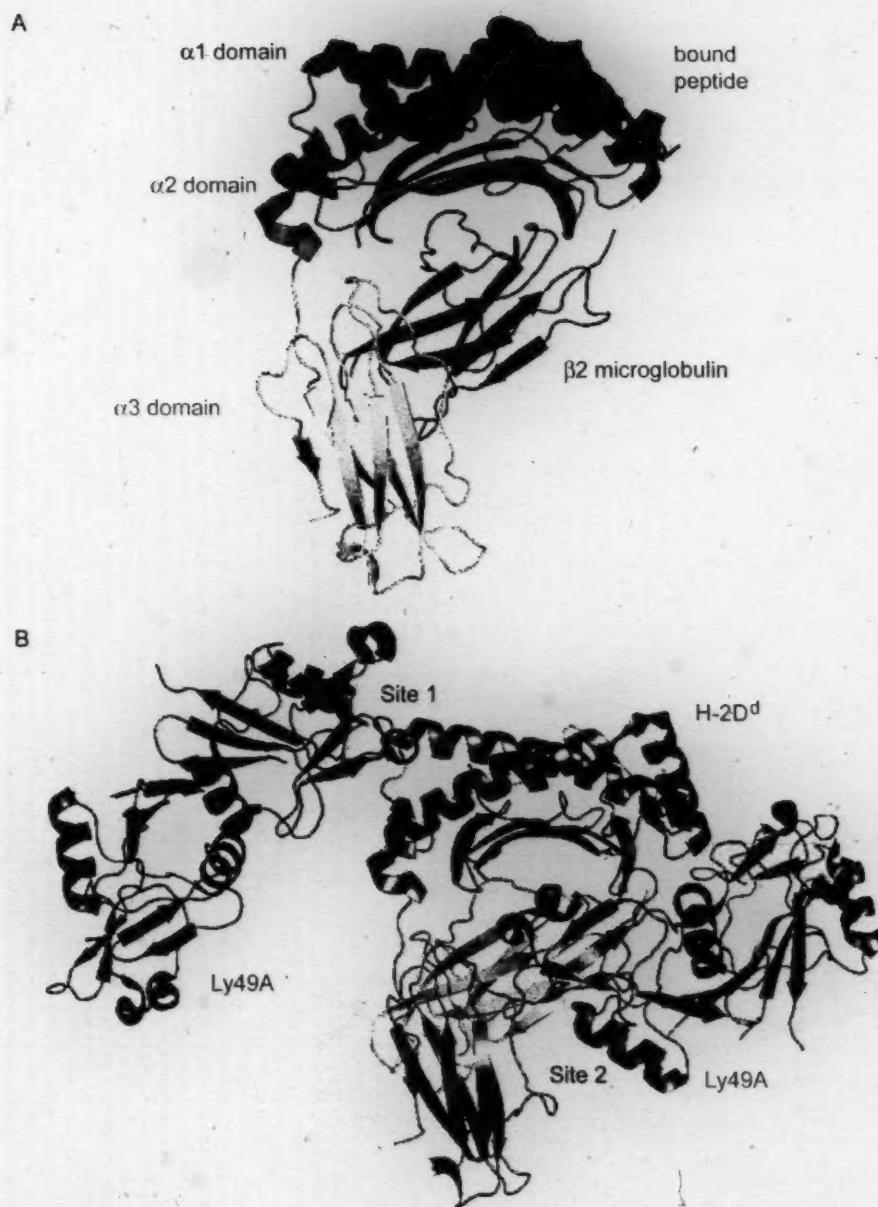


**FIGURE 1-4.** Crystal structures depicting the H-2D<sup>d</sup> MHC I molecule and its association at two distinct sites with Ly49A. A, The MHC I heavy chain is made up of three domains,  $\alpha 1$  (blue),  $\alpha 2$  (green) and  $\alpha 3$  (yellow). The heavy chain non-covalently associates with  $\beta 2$ -microglobulin (purple) and the  $\alpha 1\alpha 2$  domains form a groove that binds peptide (red spheres). B, The co-crystal of H-2D<sup>d</sup> (blue ribbon) with two Ly49A homodimers, one interacting at site 1 (purple ribbon) and one at site 2 (teal ribbon).

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## CHAPTER II

### THE RAT RT1-A1<sup>C</sup> MHC MOLECULE IS A XENOGENEIC LIGAND RECOGNIZED BY THE MOUSE ACTIVATING LY-49W AND INHIBITORY LY-49G RECEPTORS<sup>1,2</sup>

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#### A. Introduction

Natural killer cells play a significant role in innate immunity against virally infected and transformed cells (1) and contribute to transplant rejection, particularly hybrid resistance to allogeneic and xenogeneic bone marrow transplants (2, 3). Effector functions of NK cells include lysis of pathologically altered cells and the production and secretion of cytokines (4). Murine NK cells express a number of activating and inhibitory receptors that regulate these effector functions, including members of the Ly-49 receptor family (5). Ly-49 receptors belong to the C-type lectin receptor superfamily and are expressed at the cell surface as disulfide-bonded homodimers (6). These type II transmembrane proteins are encoded as a *Ly-49* multi-gene family on chromosome 6 in the NK gene complex (NKC) and inbred mouse strains can differ with respect to their complement of activating and inhibitory type *Ly-49* genes (7, 8). Inhibitory Ly-49 receptors contain immuno-receptor tyrosine-based inhibitory (ITIM) motifs in their cytoplasmic tails that become phosphorylated on tyrosine upon ligand recognition (9). The phosphorylated ITIMs recruit the Src homology 2 domain-containing tyrosine phosphatase-1, which

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disrupts membrane proximal signaling events that would otherwise lead to NK activation (10, 11). Activating Ly-49 receptors do not possess ITIMs, and instead, contain a charged residue in their transmembrane domain that facilitates association with the immuno-receptor tyrosine-based activation motif (ITAM) -containing, signaling adapter molecule, DAP12. Upon ligand engagement by the Ly-49 activating receptor, phosphorylation of tyrosine residues within the DAP12 ITAMs, leads to tyrosine kinase (Syk, Zap-70) -mediated signaling events and NK cell activation (12-15).

Ly-49 receptors are more extensively characterized in the mouse than in the rat. In both species, inhibitory Ly-49 receptors recognize classical class I MHC products, allowing NK cells to detect cells with loss of class I expression, targeting them for destruction by co-expressed activating receptors (16-21). Some activating Ly-49 receptors in the mouse recognize classical class I MHC molecules leading to target cell destruction (21-25), while rat activating Ly-49 receptors recognize non-classical class I molecules (26-28). In addition, an activating Ly-49 receptor that recognizes a mouse cytomegalovirus-encoded ortholog of a class I MHC molecule, significantly contributes to MCMV resistance (29-31).

Mouse Ly-49 receptors are also capable of recognizing xenogeneic ligands. The activating Ly-49D receptor recognizes a hamster class I molecule and an unidentified MHC-encoded rat ligand of the F344 and LEW rat strains (32, 33). Furthermore, the activating Ly-49W and inhibitory Ly-49G receptors also recognize rat strain specific xenogeneic ligands (34). Identification of xenogeneic ligands and characterization of the molecular basis of their recognition may provide additional insights into conserved or novel modes of Ly-49 receptor ligand recognition. Additionally, the identification of Ly-49 receptors and their xeno-ligands could aid studies investigating the role of NK cells in the generation and use of xenogeneic mixed chimeras for the induction of xenotransplantation tolerance (35). However, there are no reports of a specific rat xenogeneic ligand for any mouse Ly-49 receptor.

In this report we identify RT1-A1<sup>c</sup>, a rat classical class I MHC molecule, as a ligand for the closely related Ly-49W activating and Ly-49G<sup>BALB/c</sup> inhibitory receptors. This xenogeneic class I recognition is demonstrated to be allele specific, as other class I molecules of the RT1<sup>c</sup> haplotype and a classical class I molecule encoded by a different



MHC haplotype, RT1<sup>av1</sup>, are not recognized by Ly-49W and G<sup>BALB/c</sup>. Additionally, specificity for RT1-A<sup>c</sup> can be transferred from Ly-49W to Ly-49P by substitution of three residues shared by Ly-49W and G<sup>BALB/c</sup> but not Ly-49P. These residues are located in the Ly-49  $\beta$ 4- $\beta$ 5 loop which participates in mouse Ly-49 receptor interactions with mouse class I ligands (36), suggesting that mouse Ly-49 recognition of rat class I molecules follows similar principles of interaction. Preliminary analysis of conserved motifs shared by RT1-A1<sup>c</sup> and mouse ligands of Ly-49W and G<sup>BALB/c</sup> implicate a conserved MHC supertype motif as a potential mediator of xenogeneic and syngeneic recognition.

## **B. Materials and Methods**

### *Animals*

Five to 8 week old female DBA/2J (H-2<sup>d</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Six to 8 week old male and female PVG (RT1<sup>c</sup>) and DA (RT1<sup>av1</sup>) rats were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and Harlan U.K. (Bicester, U.K.). Male and female intra-MHC recombinant rat strains PVG.R1, PVG.R19 and PVG.R20 were purchased at 6 to 8 weeks of age from Dr. G. Butcher at The Babraham Institute (Cambridge, UK). All animals were housed in approved animal care facilities in accordance with institutional guidelines.

### *Hybridomas and antibodies*

The Cwy-3 (IgG1), 4D11 (rat IgG2a) and A1 (IgG2a) anti-Ly-49 hybridomas have been described (37-39). Isotype controls include BB7.1 (IgG1) anti-HLA-B7 (40), 53-5.8.3 (rat IgG2a) anti-murine Lyt-3 (41) and B27M1 (IgG2a) anti-HLA-B27, B7 (42). The Cwy-3 hybridoma was generated in this laboratory, the 53-5.8.3 hybridoma kindly provided by Dr. L.A. Herzenberg (Stanford University, Stanford CA) and all other hybridomas were obtained from American Type Culture Collection (Manassas, VA). Antibodies were prepared from ammonium sulfate precipitates as described (24).

### *Cell lines*

YB2/0, a non-secreting rat myeloma, was obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% FCS, L-glutamine,

penicillin, streptomycin, 1 mM sodium pyruvate and 0.1mM non-essential amino acids. RNK-16, a spontaneous F344 rat strain NK cell leukaemia cell line (43), was provided by Dr. M. Nakamura at the University of California (San Francisco, CA), and maintained in RPMI supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and  $5 \times 10^{-5}$  M 2-mercaptoethanol. RNK-16 effector cells expressing Ly-49 receptors were generated by this lab as described (24, 25, 34) and maintained under G418 selection until 48 hours prior to cytotoxicity assays.

#### *Mutagenesis of Ly-49W and Ly-49P*

Ly-49W and Ly-49P, previously cloned in this laboratory from the NOD mouse (24, 25) were mutated using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA). Mutagenic primers were designed to mutate amino acid residues N245, D247 and Q248 in Ly-49P to the corresponding residues found in Ly-49W, D250, G252 and K253. Inversely, residues D250, G252 and K253 in Ly-49W were mutated to the Ly-49P residues, N245, D247 and Q248. All mutations were verified by DNA sequencing. The cDNAs for the coding regions of the mutant Ly-49 constructs were inserted into the *XhoI/XbaI* sites of the mammalian expression vector BSR $\alpha$ EN (provided by Dr. A. Shaw, Washington University, St. Louis, MO). RNK-16 cells were transfected with each construct as previously described (25), and maintained under G418 selection until 48 hours prior to cytotoxicity assays.

#### *RNA preparation and cDNA synthesis*

Spleens harvested from PVG and DA rats were immediately immersed in RNAlater RNA stabilization reagent (Qiagen Inc., Valencia, CA), homogenized using a rotor-stator homogenizer and total RNA isolated using an RNeasy Protect Mini Kit (Qiagen Inc.). cDNA was produced using Powerscript reverse transcriptase (BD Clontech, Palo Alto, CA) with an oligo(dT) primer.

#### *Generation of YB2/0 target cells expressing rat class I MHC-EGFP fusion proteins*

H-2D<sup>k</sup>, previously cloned in this laboratory (34) was directionally cloned, in frame, into the *XhoI/XmaI* sites of pEGFP-N2ML (provided by Dr. D. Burshtyn,

University of Alberta, Edmonton, Canada). The pEGFP-N2ML vector has the EGFP initiation codon mutated to ATA, creating a C-terminal EGFP-fusion protein. As the majority of published rat class I MHC sequences do not include the leader sequence, silent mutations (QuikChange, Stratagene) creating an *MluI* site at the leader/coding region junction of H-2D<sup>k</sup> allowed cloning of rat class I MHC coding sequences fused to this mouse class I leader sequence. Each rat class I MHC transcript, without leader sequence, was amplified with Advantage-HF 2 polymerase mix, digested with *MluI/XmaI* and ligated into the H-2D<sup>k</sup> leader-EGFP fusion vector. Successful generation of in frame H-2D<sup>k</sup> leader - rat class I MHC-EGFP constructs was verified by DNA sequencing. YB2/0 cells were transfected with each construct as previously described (24) and maintained under G418 selection until 48 hours prior to cytotoxicity assays.

#### *Flow cytometric analysis*

Expression of rat class I MHC constructs in YB2/0 cells was determined by EGFP fluorescence intensity relative to untransfected YB2/0 cells using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Expression of mouse Ly-49 receptors on RNK-16 transfectants was monitored with Cwy-3, 4D11 and A1 antibodies as previously described (24, 25, 34).

#### *Generation of Con A T cell blasts*

Con A-activated T cell blasts were prepared from spleens of DBA/2 mice and PVG and DA rat strains. Spleen cells were cultured at  $5 \times 10^6$  cells/ml in RPMI 1640 supplemented with 10% FCS, 2-mercaptoethanol and 3 µg/ml Con A (Sigma-Aldrich, St. Louis, MO) for 48 hours. Blast cells were recovered after washing in RPMI 1640 medium. No difference in blast formation or RNK-16 effector recognition was observed between spleen cells of male or female origin.

#### *Cytotoxicity assays*

Target cells were labeled with 100-150 µCi of Na<sup>51</sup>CrO<sub>4</sub> (<sup>51</sup>Cr) (Mandel, Guelph, Canada) at 37°C for either 1 hour (YB2/0 and YB2/0 transfectants) or 1.5 hours (Con A blasts). Targets were washed extensively and  $1 \times 10^4$  <sup>51</sup>Cr-labeled cells were incubated



with RNK-16 or transfected RNK-16 cells for 4 hours at 37°C in V-bottom microtitre plates at various E:T ratios in triplicate. After incubation, supernatant samples were collected and counted in a MicroBeta TriLux liquid scintillation counter (PerkinElmer, Wellesley, MA). Percent specific lysis was determined as (experimental release – spontaneous release)/(maximal release – spontaneous release) x 100%. In Ly-49 specific antibody-blocking assays, mAbs were incubated for 30 minutes with 1 µg of soluble Protein A/G (Calbiochem, La Jolla, CA) for each µg of mAb before addition to effector cells to prevent reverse antibody-dependent cell-mediated cytotoxicity (rADCC). Effector cells were incubated with the mAb-Protein A/G mixture for 30 minutes prior to addition of target cells. All cytotoxicity experiments were repeated a minimum of three times.

### C. Results

*Con A blasts from the PVG rat strain are recognized by Ly-49W and the ectodomain of the BALB/c allele of Ly-49G*

Ly-49D and Ly-49G<sup>BALB/c</sup> recognize hamster and rat xenogeneic ligands (32-34). Features that are shared by or distinguish xenogeneic and mouse ligands may offer novel insights into the molecular basis of Ly-49 ligand recognition. Additionally, the identification of Ly-49 receptors and their xenogeneic MHC ligands could serve in the construction of xenogeneic mixed chimeras that do not require depletion of host NK cells in xenotransplantation tolerance models (44). Ly-49D recognizes a xenogeneic ligand on chinese hamster ovary (CHO) cells (33, 45), which has been identified as the class I MHC molecule Hm1-C4 (32). As a xenogeneic ligand, Hm1-C4 has offered limited insight into Ly-49 recognition motifs, primarily due to the phylogenic distance between hamster and mouse, resulting in relatively low sequence identity between their respective class I MHC molecules (32). Furthermore, hamsters are not used in studies of xenogeneic mixed chimerism. In contrast, the rat is more closely related to mouse, expresses class I MHC molecules that are relatively high in sequence identity to mouse MHC while displaying evolutionary distance, and rat/mouse xenogeneic mixed chimeras are a traditional model within transplantation studies. These properties make rat a more informative partner to mouse in which to examine shared recognition motifs across species. The Ly-49D receptor mediates cytolysis of Con A blasts from both the F344 and



LEW rat strains in an MHC haplotype-specific manner (33), but no specific xenogeneic ligand has been identified. Our laboratory has also demonstrated differential recognition of xenogeneic Con A blasts from the PVG and LEW rat strains by the Ly-49G inhibitory receptor (34). As recognition of rat Con A blasts by mouse Ly-49 receptors is strain specific, and no rat xenogeneic ligands have been definitively identified for mouse Ly-49 receptors, we set out to identify rat ligand(s) mediating the strain specific recognition by Ly-49G<sup>BALB/c</sup> and the related Ly-49W activating receptor.

RNK-16 cells are an NK leukemia cell line derived from the F344 rat strain and express Ly49 receptors found in this strain (46-48) but are not intrinsically cytolytic toward Con A blast targets from rat strains of the RT1<sup>c</sup> and RT1<sup>av1</sup> MHC haplotypes (Fig. 2-1A). RNK-16 cells were therefore transfected with mouse Ly-49 receptors and tested for gain of cytotoxicity toward Con A blasts prepared from rat strains expressing these MHC haplotypes, PVG (RT1<sup>c</sup>) and DA (RT1<sup>av1</sup>). The specificity of the Ly-49G<sup>BALB/c</sup> inhibitory receptor for Con A blasts was examined through the use of a chimeric receptor, Ly-49WG<sup>BALB/c</sup>, described previously (34). This chimeric activating receptor maintains the specificity of the Ly-49G<sup>BALB/c</sup> inhibitory receptor ectodomain, while possessing the transmembrane and cytosolic portions of the activating receptor Ly-49W. As previously demonstrated, RNK-16 cells transfected with the chimeric Ly-49WG<sup>BALB/c</sup> receptor, specifically recognize and lyse Con A blasts prepared from the PVG rat strain, which expresses MHC molecules of the RT1<sup>c</sup> haplotype (Fig. 2-1B). Two different activating Ly-49 receptors originally cloned from the NOD mouse, Ly-49P and Ly-49W (24, 25), were also tested for their ability to trigger lysis of xenogeneic rat Con A blasts when transfected into RNK-16 cells. The ectodomain of Ly-49P closely resembles that of the inhibitory receptor Ly-49A, while that of Ly-49W resembles that of the inhibitory Ly-49G receptor. Ly-49W was also seen to trigger cytolysis of Con A blasts specifically from the PVG rat strain (Fig. 2-1C). In contrast, Ly-49P was incapable of triggering cytolysis of blasts from the PVG rat strain, despite being competent in triggering lysis of Con A blasts from the control DBA/2 mouse strain (H-2<sup>d</sup>) (Fig. 2-1D) (24). Thus, Ly-49G and Ly-49W, which share 97% amino acid identity in their ectodomains, recognize PVG strain rat ligand(s) while the Ly-49A-related Ly-49P does not.

*RT1<sup>av1/c</sup> intra-MHC recombinants identify the classical class I region of the RT1<sup>c</sup> haplotype as essential for recognition by Ly-49W and Ly-49WG<sup>BALB/c</sup>*

Since xenogeneic recognition of rat targets by mouse Ly-49 receptors occurs in both a strain (33, 34) and an MHC haplotype-specific manner (33), we aimed to determine if the xenogeneic recognition of PVG rat strain by Ly-49W and Ly-49G<sup>BALB/c</sup> could be attributed to a specific region of the major histocompatibility complex within this strain.

The rat major histocompatibility complex is encoded on chromosome 20 (49). It contains a classical class I MHC encoding region, RT1-A, a class II MHC encoding region, RT1-B/D, and a non-classical class I MHC encoding region, RT1-C/E/M (Fig. 2-2A). The RT1-A region of the RT1 complex encodes between one and three classical class I MHC molecules, depending on the rat strain. In the PVG rat (haplotype c), the RT1-A region encodes two molecules, RT1-A1<sup>c</sup> and RT1-A2<sup>c</sup>, whereas the DA rat strain (haplotype av1) only encodes one molecule in this region, RT1-A<sup>a</sup> (Fig. 2-2B) (50). The non-classical class I MHC region, RT1-C/E/M, encodes numerous molecules, many of which have yet to be fully characterized. The more centromerically encoded molecules in this region, are known to be classical-like in both structure and function (49, 51). Of these classical-like molecules, some have been cloned from the PVG (Genbank accession: CAD60946, CAA06296, CAA74192) and DA rat strains (Genbank accession: CAD60945) (Fig. 2-2B).

Both the classical RT1-A and non-classical RT1-C/E/M regions of the PVG rat are known to encode molecules recognized by the rat Ly-49 receptors, Ly49i2 and Ly49s3, respectively (18, 26). Therefore, we wished to examine each region separately to determine whether the recognition of PVG Con A blasts by Ly-49W and Ly-49WG<sup>BALB/c</sup>, could be attributed to molecules encoded in either the RT1-A region or RT1-C/E/M region of the PVG rat. We obtained PVG congenic, intra-MHC recombinant rat strains between the non-recognized RT1<sup>av1</sup> haplotype and the recognized RT1<sup>c</sup> haplotype (Dr. G. Butcher, Babraham Institute, Cambridge, UK) that have a recombination between classical class I and class II regions, or class II and non-classical class I regions (Fig. 2-2B). These recombinants allowed us to examine the requirement for the RT1<sup>c</sup> haplotype

at each RT1 region, for recognition and lysis of targets by RNK-16 cells expressing Ly-49W and Ly-49WG<sup>BALB/c</sup>. Untransfected RNK-16 cells were unable to mediate cytolysis of Con A blast targets from any of the recombinant strains (Fig. 2-3A). RNK-16 cells transfected with the activating Ly-49W receptor mediated cytolysis of the PVG.R20 (*c-c-a*) recombinant strain but not the PVG.R1 (*a-c-c*) or PVG.R19 (*a-a-c*) intra-MHC recombinants (Fig. 2-3B), indicating that a molecule encoded in the classical class I RT1<sup>c</sup> region is required for recognition by Ly-49W. Similarly, RNK-16 cells expressing the Ly-49WG<sup>BALB/c</sup> activating chimera were also seen to mediate cytolysis of Con A blasts from the PVG.R20 (*c-c-a*) strain which expresses the *c* haplotype in the classical class I region, but not of Con A blasts from either PVG.R19 (*a-a-c*) or PVG.R1 (*a-c-c*) strains (Fig. 2-3C), which only express the *c* haplotype in the non-classical class I region or non-classical class I and class II MHC regions, respectively. Therefore, both the Ly-49W receptor and Ly-49WG<sup>BALB/c</sup> activating chimera recognize a ligand(s) encoded in the classical class I region of the RT1<sup>c</sup> haplotype.

*The classical rat class I MHC molecule, RT1-A1<sup>c</sup>, is a xenogeneic ligand for the murine Ly-49W and Ly-49G<sup>BALB/c</sup> receptors*

Since xenogeneic recognition by Ly-49W and Ly-49WG<sup>BALB/c</sup> was dependent on expression of the *c* haplotype at the classical class I region of the RT1 complex in our recombinant studies, we examined whether recognition was mediated specifically by one of the two classical class I molecules encoded in the RT1-A region of the PVG rat, either RT1-A1<sup>c</sup> or RT1-A2<sup>c</sup> (Fig. 2-2B).

Rat MHC molecules were cloned from splenic cDNA libraries of DA and PVG strain rats. As specific antibodies are not available to all of the MHC molecules assayed, each molecule was expressed as a C-terminally fused EGFP protein. The fusion of EGFP to the cytoplasmic tail of each MHC molecule allowed detection of expression through green fluorescence, while conserving the extracellular domain structure for examination of xeno-recognition by mouse Ly-49 receptors. Three MHC-EGFP fusion constructs were prepared from the PVG rat strain: the two classical class I molecules RT1-A1<sup>c</sup> and RT1-A2<sup>c</sup> as well as the non-classical class I molecule, RT1-U2<sup>c</sup>. An MHC-EGFP fusion construct of the classical class I molecule RT1-A<sup>a</sup> was also prepared from the control DA

rat strain which does not have a ligand for Ly-49W or Ly-49G<sup>BALB/c</sup>. Each of the MHC-EGFP fusion constructs were transfected into the rat myeloma cell line, YB2/0 (RT1<sup>u</sup>) and expression was determined by EGFP fluorescence intensity relative to untransfected YB2/0 cells (Fig. 2-4).

Transfection of YB2/0 cells with the classical class I region encoded MHC molecule, RT1-A1<sup>c</sup>, dramatically increased lysis by RNK-16 cells transfected with either Ly-49W or Ly-49WG<sup>BALB/c</sup> (Fig. 2-5A-C). Lysis of RT1-A1<sup>c</sup> transfected YB2/0 cells was specific, as YB2/0 cells expressing the other classical class I region encoded MHC molecule found in the *c* haplotype, RT1-A2<sup>c</sup>, were not lysed above background levels by either Ly-49W or Ly-49WG<sup>BALB/c</sup> transfected RNK-16 cells (Fig. 2-5A). As expected, YB2/0 cells expressing a non-classical class I MHC molecule from the PVG rat, RT1-U2<sup>c</sup>, were not recognized by Ly-49W or Ly-49WG<sup>BALB/c</sup> expressing RNK-16 cells (Fig. 2-5B). Additionally, YB2/0 cells expressing RT1-A<sup>a</sup>, from the DA rat strain, did not trigger lysis through recognition by Ly-49W or Ly-49WG<sup>BALB/c</sup> (Fig. 2-5C).

We wanted to ensure that recognition of RT1-A1<sup>c</sup> transfected YB2/0 cells by the activating chimera Ly-49WG<sup>BALB/c</sup> is also mediated by the native Ly-49G<sup>BALB/c</sup> inhibitory receptor. RNK-16 cells and RNK-16 cells transfected with inhibitory Ly-49 exhibit moderate background cytolysis of untransfected YB2/0 cells. This is in contrast to RNK-16 cells transfected with activating Ly-49, which exhibit very low or no background cytolysis of untransfected YB2/0 cells, possibly due to the sequestration of DAP12 by activating Ly-49 from the unidentified endogenous activating receptor mediating the cytotoxicity. The background cytolysis of YB2/0 cells by RNK-16 cells expressing Ly-49G<sup>BALB/c</sup> is completely inhibited when YB2/0 cells are transfected with RT1-A1<sup>c</sup> but not with other class I molecules from the PVG and DA rat strains (Fig. 2-6A). The 4D11 antibody, which recognizes Ly-49G<sup>BALB/c</sup>, reverses the inhibition of the Ly-49G<sup>BALB/c</sup> expressing RNK-16 transfectants by RT1-A1<sup>c</sup>, whereas the isotype control antibody does not (Fig. 2-6B). These results demonstrate that, like the chimeric receptor, Ly-49WG<sup>BALB/c</sup>, the native inhibitory Ly-49G<sup>BALB/c</sup> receptor mediates specific recognition of RT1-A1<sup>c</sup>. Thus expression of individual RT1 molecules from the recognized PVG rat strain and non-recognized DA rat strains in YB2/0 cells allowed identification of the classical class I MHC molecule, RT1-A1<sup>c</sup>, as a xenogeneic ligand capable of triggering



NK cell effector functions through recognition by the mouse Ly-49 receptors Ly-49W and Ly-49G<sup>BALB/c</sup>.

*Mutagenesis of Ly-49P  $\beta$ 4- $\beta$ 5 loop residues to corresponding residues of Ly-49W and Ly-49G<sup>BALB/c</sup> receptors confers specificity for a ligand in the PVG rat*

Analysis of the Ly-49A/H-2D<sup>d</sup> co-crystal (52) implicates participation of Ly-49  $\beta$ 4- $\beta$ 5 loop residues, among others, in class I MHC recognition. Furthermore, non-conserved residues in this loop may be responsible for the specificity of Ly-49 receptors for particular class I alleles (34). Since we observed class I allele specific rat xenogeneic ligand recognition by Ly-49W and G<sup>BALB/c</sup>, we tested for the potential participation of the Ly-49  $\beta$ 4- $\beta$ 5 loop in determining specificity for the rat RT1-A1<sup>c</sup> class I ligand. Both Ly-49W and Ly-49G<sup>BALB/c</sup> contain the residues DCGK in their predicted  $\beta$ 4- $\beta$ 5 loop, whereas Ly-49P contains the residues NCDQ in this region. To determine the involvement of DCGK in recognition of PVG Con A blasts by Ly-49W, we mutated the Ly-49P receptor, which does not recognize the PVG rat strain (Fig. 2-7A), to express  $\beta$ 4- $\beta$ 5 loop residues from Ly-49W and saw a gain of PVG rat strain target recognition by the mutant Ly-49P receptor (Fig. 2-7B). Conversely, when we mutated the Ly-49W receptor, which recognizes the PVG rat strain (Fig. 2-7C), to express residues found in the  $\beta$ 4- $\beta$ 5 loop of Ly-49P, we saw no loss of PVG Con A blast recognition by the mutant Ly-49W receptor (Fig. 2-7D).

Although the wild-type Ly-49P receptor does not recognize any ligand from RT1<sup>av1/c</sup> intra-MHC recombinant rat strains (Fig. 2-8A), the gain of function mutant Ly-49P receptor demonstrated recognition of a ligand localized to the RT1-A region of the rat c haplotype (Fig. 2-8B). In contrast, when comparing the recognition of the wild-type form of the Ly-49W receptor (Fig. 2-8C) to mutant Ly-49W (Fig. 2-8D), both recognized a ligand in the RT1-A region of the rat c haplotype, indicating no loss of function for the Ly-49W  $\beta$ 4- $\beta$ 5 loop mutant. We could not confirm RT1-A1<sup>c</sup> as opposed to RT1-A2<sup>c</sup> as the recognized ligand due to high background lysis of YB2/0 RT1-A1<sup>c</sup> and A2<sup>c</sup> transfectants by the mutant Ly-49 RNK-16 transfectants (data not shown). Nevertheless, a classical class I ligand of the c haplotype is recognized and is most likely RT1-A1<sup>c</sup> considering our results using wild-type Ly-49W and G<sup>BALB/c</sup> receptors (Fig. 2-5). These

results indicate that specificity for xenogeneic rat class I can be transferred by substitution with specific residues from the  $\beta 4$ - $\beta 5$  loop of Ly-49W. They also reveal that these residues can not be solely responsible for xenogeneic ligand specificity, as the Ly-49W  $\beta 4$ - $\beta 5$  loop mutant retains its recognition of a PVG ligand.

#### D. Discussion

In this study, we demonstrate that the rat class I MHC molecule RT1-A1<sup>e</sup> is a xenogeneic ligand for the Ly-49W activating and Ly-49G<sup>BALB/c</sup> inhibitory receptors, a surprising result given that approximately 20 million years have transpired since the evolutionary divergence of mice and rats. Furthermore, rodent class I MHC and Ly-49 genes are evolving rapidly with significant species-specific evolution, resulting in no evident class I MHC orthologs (53), nor clear rat orthologs of Ly-49W or G<sup>BALB/c</sup> (Fig. 2-9). All known rat Ly-49 receptors have at most 67% amino acid identity with the mouse Ly-49W and G<sup>BALB/c</sup> receptors. The rat Ly-49i2, an inhibitory receptor, also recognizes RT1-A1<sup>e</sup> (18), but it is distantly related to Ly-49W and G<sup>BALB/c</sup> (Fig. 2-9), sharing only 49-50% amino acid identity. With the absence of a clear Ly-49 or MHC ortholog between mouse and rat and their relatively low sequence identity, an alternative explanation appears necessary for this xenorecognition besides simple non-divergence of sequence. One possibility would be the convergent evolution of MHC through common selective pressures between these two species, a phenomenon previously demonstrated between the MHC of mice and more distantly related primates for T cell recognition (54). The convergent evolution of class I MHC may have resulted in RT1-A1<sup>e</sup> recognition as a syngeneic ligand by rat Ly49i2 and as a xenogeneic class I ligand by unrelated Ly-49 receptors, Ly-49W and G<sup>BALB/c</sup>, of a different rodent species.

It is interesting to consider whether the molecular basis of Ly-49W and G<sup>BALB/c</sup> recognition of RT1-A1<sup>e</sup> resembles Ly-49 recognition of mouse class I molecules. The co-crystal of Ly-49A bound to H-2D<sup>d</sup> serves as a model for mouse Ly-49-class I interactions. Two independent sites of Ly-49A interaction with H-2D<sup>d</sup> are identified: site 1 at the N-terminus of the  $\alpha 1$  helix and C-terminus of the  $\alpha 2$  helix, and site 2 in the cleft bordered by the  $\alpha 1/\alpha 2$  domains, the conserved  $\alpha 3$  domain and  $\beta 2$ -microglobulin (52). We demonstrate that Ly-49W and Ly-49G<sup>BALB/c</sup>, but not Ly-49P, recognize RT1-A1<sup>e</sup> and a

previous report indicates that Ly-49D does not recognize PVG strain ligands including RT1-A1<sup>c</sup> (33). Since Ly-49A, D, G, P, and W share significant sequence identities and belong to the Ly-49A-G subfamily (55), the differences in class I recognition are presumed to involve polymorphic residues of the Ly-49 receptors, particularly in the ligand binding C-terminal lectin-like domain. Using the Ly-49A/H-2D<sup>d</sup> co-crystal for modeling mouse Ly-49 interactions with RT1-A1<sup>c</sup>, indicates that polymorphic residues of Ly-49 receptors are concentrated in the  $\beta$ 4- $\beta$ 5 loop, which can contact class I at either site 1 or site 2, and thus are candidates for determining xenogeneic class I specificity. Ly-49W and G<sup>BALB/c</sup> share the sequence DCGK at the  $\beta$ 4- $\beta$ 5 loop and recognize RT1-A1<sup>c</sup>, whereas Ly-49D is YCDQ and Ly-49P is NCDQ at this location. Interestingly, the distantly related rat Ly-49i2 receptor, which like Ly-49W and G<sup>BALB/c</sup>, recognizes RT1-A1<sup>c</sup> (18), also bears the DCGK sequence at its predicted  $\beta$ 4- $\beta$ 5 loop. We demonstrate that transfer of specificity for an RT1-A<sup>c</sup> molecule can be conferred to a mutant Ly-49P receptor by simply substituting the  $\beta$ 4- $\beta$ 5 loop residues common to Ly-49W, G<sup>BALB/c</sup> and i2 for those in wild-type Ly-49P. This result suggests that these residues can confer RT1-A1<sup>c</sup> specificity, perhaps by strengthening interactions with class I residues at site 1 or 2, or alternative site(s), exceeding a functional threshold. However, the DCGK sequence is not solely responsible for RT1-A<sup>c</sup> specificity, as mutating the Ly-49W receptor to express the  $\beta$ 4- $\beta$ 5 residues from Ly-49P does not affect the recognition of RT1-A<sup>c</sup> by Ly-49W. These findings indicate that  $\beta$ 4- $\beta$ 5 residues, DCGK in particular, can influence the specificity of Ly-49 receptors for xenogeneic class I, but contributions of other Ly-49 residues are also significant to recognition.

We demonstrate that recognition of xenogeneic rat class I molecules by mouse Ly-49W and G<sup>BALB/c</sup> receptors is specific, observing interactions with RT1-A1<sup>c</sup>, but not RT1-A<sup>a</sup> or RT1-A2<sup>c</sup>. An examination of amino acid sequence differences between RT1-A1<sup>c</sup>, RT1-A<sup>a</sup> and RT1-A2<sup>c</sup> may provide insights into the differential recognition of the rat class I molecules. Only a few residues, modeled to be involved in site 1 or 2 interactions, differ between RT1-A1<sup>c</sup> and the other two, rat class I molecules. All site 2 residues on the rat class I molecules are conserved and thus do not offer an obvious explanation for the differential interactions observed. RT1-A2<sup>c</sup> and RT1-A<sup>a</sup> both differ from RT1-A1<sup>c</sup> at site 1, with RT1-A2<sup>c</sup> having Q50 and H174 and RT1-A<sup>a</sup> having S169 compared with R50,



L174 and R169 in RT1-A1<sup>c</sup>. The site 1 residues differing in RT1-A2<sup>c</sup> and RT1-A<sup>a</sup> also differ from those at the same positions in the mouse class I ligands of Ly-49W and G<sup>BALB/c</sup>. These differences at site 1 may account for the differential recognition of rat class I molecules; however, mutagenesis experiments will be necessary to substantiate this possibility. Additionally, outside of the site 1 and site 2 interfaces, RT1-A2<sup>c</sup> and RT1-A<sup>a</sup> have several additional non-conservative amino acid substitutions that may contribute to the differences in class I specificity.

The absence of amino acid differences at site 2 and limited differences at site 1, between RT1-A1<sup>c</sup> and RT1-A<sup>a</sup> or RT1-A2<sup>c</sup>, suggest additional influences on mouse Ly-49W and G<sup>BALB/c</sup> specificity for xenogeneic class I ligands. Observations in humans and subsequently in other species, indicate that class I molecules can be categorized into a limited number of "supertypes" based on their preferences for specific anchor residues in peptide binding (56-59). In humans, although class I alleles exhibit extensive diversity, they can be grouped into only nine supertypes (60) when the variation of anchor residues is considered. Supertype groupings also extend to class I molecules of other, even distantly related, species. For example, mouse and humans share several supertypes between non-orthologous class I genes, suggesting convergent evolution of supertypes, (54, 61) presumably due to a need in multiple species to possess class I molecules that collectively have the ability to bind peptides with the same 10 or fewer distinct patterns of anchor residues. The HLA-B7 supertype typically requires peptides to have proline at the second, or P2, position and a hydrophobic C-terminus (62). It is worthy of note that most of the rodent class I molecules that are recognized by Ly-49W and/or Ly-49G<sup>BALB/c</sup> have this B7 supertype (H-2L<sup>d</sup> and RT1-A1<sup>c</sup>) (62) or prefer a P2 residue with a small side chain (H-2D<sup>d</sup> prefers G at P2 and P at P3) (62). In contrast, all rat or mouse class I molecules not recognized by Ly-49W or G<sup>BALB/c</sup> (RT1-A<sup>a</sup>, H-2K<sup>k</sup>, K<sup>d</sup>, K<sup>b</sup> and D<sup>b</sup>), belong to other supertypes with distinct anchor residues (62). One exception to this pattern is H-2D<sup>k</sup>, which does not have a B7 supertype (62) but is recognized by Ly-49W and G<sup>BALB/c</sup>. RT1-A2<sup>c</sup> is not included in this comparison since the peptide anchor residues it prefers have not been reported. These relationships raise the possibility that Ly-49W and G<sup>BALB/c</sup>, along with related receptors, may recognize a conformational epitope on class I that is influenced by peptide anchor residues and have evolved to survey cells for class I MHC



molecules of, or related to, the HLA-B7 supertype. Potentially, the shared recognition of RT1-A1<sup>c</sup> by the rat Ly49i2 and mouse Ly-49W and G<sup>BALB/c</sup> receptors is due to similar evolutionary pressures and convergent evolution, leading to a common requirement for murine NK cell recognition of the HLA-B7/RT1-A1<sup>c</sup>/H-2L<sup>d</sup> supertype.

Although the role of bound peptide is unknown in xenogeneic rat class I recognition by Ly-49 receptors, we considered the potential influence of TAP2 polymorphism in the differential recognition of rat class I molecules by mouse Ly-49W and G<sup>BALB/c</sup>, and deemed it unlikely. Two versions of the heterodimeric transporter associated with antigen processing, TAP-A and TAP-B, are expressed in the rat due to the existence of two alleles of *TAP2* in this species (63, 64). The specificity for peptide transport differs between TAP-A and TAP-B and only one or the other *TAP2* allele is expressed in each inbred rat strain (65). TAP-A, like human TAP, exhibits broad specificity, transporting peptides with either hydrophobic or positively charged C-terminal residues for assembly with class I. In contrast, TAP-B displays a narrower specificity and strong bias for peptides with hydrophobic or aromatic C-termini, similar to mouse TAP (66). RT1-A<sup>a</sup> has a strong preference for binding peptides with a C-terminal arginine and is typically co-expressed with TAP-A, which efficiently delivers such peptides (67). In contrast, RT1-A1<sup>c</sup> prefers peptides with a C-terminal hydrophobic or aromatic residue and is typically co-expressed with TAP-B, which offers compatible peptides (67). Our studies indicate that Ly-49W and G<sup>BALB/c</sup> recognize the TAP-B dependent RT1-A1<sup>c</sup>, but not TAP-A dependent RT1-A<sup>a</sup> when expressed by transfection in YB2/0 cells. Since YB2/0 (RT1<sup>u</sup>) cells express TAP-B, it might be argued that the lack of RT1-A<sup>a</sup> recognition could be due to the absence of a co-expressed TAP-A. However, we also do not detect lysis of DA strain Con A blasts, which co-express RT1-A<sup>a</sup> and TAP-A. Thus, the differential recognition of rat class I molecules by the mouse Ly-49 molecules cannot be accounted for by a diminished supply of relevant peptides for RT1-A<sup>a</sup> binding. Whether RT1-A1<sup>c</sup> specifically requires TAP-B co-expression for Ly-49 recognition, cannot be discerned from the assortment of inbred strains available for this study. It is worth noting that the differential selectivity of peptide transport by TAP-A and TAP-B is limited to differences at the peptide C-termini and there is no additional bias with respect to transport of peptides with a proline at P2, as is preferred by RT1-A1<sup>c</sup>.

The induction of tolerance to donor antigens in adult transplantation would prevent transplanted tissue rejection. One potentially promising approach to achieve this goal under study in animal models is the generation of mixed chimeras (44). In mixed chimeras, adult animals are treated to eliminate mature host T cells, yet preserve other host hematopoietic cells, and are then reconstituted with donor bone marrow cells. Donor and host hematopoietic cells in mixed chimeras repopulate the thymus and delete both donor reactive and host reactive T cells, resulting in peripheral tolerance to donor and host tissue. Xenogeneic mixed chimeras can be generated in mice with rat hematopoietic donor cells (68) and this system serves as a model for induction of tolerance to xenogeneic transplants in adult animals (44). These rodent xenotransplantation models may aid in the understanding of more disparate xenotransplantation models such as pig and human. Mouse natural killer cells play a significant role in resisting engraftment of rat hematopoietic cells and the establishment of xenogeneic mixed chimeras (35). It is suggested that this might occur because mouse NK cell inhibitory receptors may not cross-react with xenogeneic rat class I MHC proteins and thus do not offer protection from NK cell elimination of rat donor cells (35). In this report, we demonstrate for the first time that a mouse inhibitory receptor (Ly-49G) expressed by a common mouse strain (BALB/c) can recognize a xenogeneic rat class I MHC molecule (RT1-A1<sup>c</sup>) resulting in functional inhibition. Identification of a rat class I ligand for Ly-49G and the activating Ly-49W receptor may begin to facilitate the rational design of studies to elucidate the role of specific NK cell receptors and NK cell subsets in xenogeneic bone marrow graft tolerance and rejection.

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### **F. Author Contribution to Data**

Kerry Lavender harvested all rat and mouse tissues, produced the RNA and cDNA libraries from PVG and DA strain rats, cloned RT1-A1<sup>c</sup>, A2<sup>c</sup>, U2<sup>c</sup> and A<sup>a</sup>, generated the D<sup>k</sup> leader/class I MHC/EGFP fusion constructs, generated all YB2/0 cells expressing rat class I MHC and con-A blast target cells, performed all FACS analysis, performed all cytotoxicity and blocking assays using RNK-16 cells expressing wild-type Ly-49 receptors and most assays with RNK-16 cells expressing mutant Ly-49 receptors. Brian Ma generated the RNK-16 cells expressing mutant Ly-49 receptors and performed the cytotoxicity assay in figure 2-7. Elizabeth Silver generated the RNK-16 cells expressing wild type Ly-49 receptors and the YB2/0 cells expressing H-2D<sup>d</sup>.

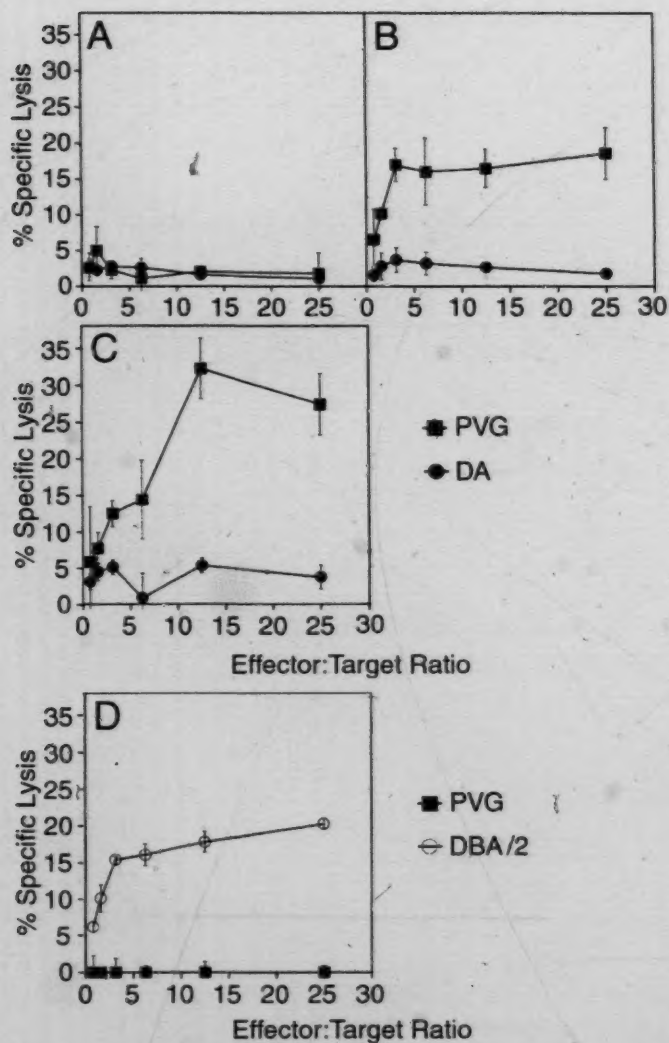


**Figure 2-1.** Xenogeneic recognition of Con A blasts from the PVG rat strain by the mouse Ly-49W activating receptor and Ly-49W<sup>BALB/c</sup> activating chimera. Con A blasts prepared from PVG (RT1<sup>c</sup>) or DA (RT1<sup>av1</sup>) rat strains were used as targets in 4-h chromium release assays with RNK-16 cells (A), RNK-16 cells transfected with the activating chimera Ly-49W<sup>BALB/c</sup> (B), or the Ly-49W activating receptor (C). Con A blasts prepared from PVG rat strain and the positive control mouse strain DBA/2 (H-2<sup>d</sup>) were targets for RNK-16 cells transfected with the Ly-49P activating receptor (D). Data represent the mean of triplicate wells  $\pm$  SD.



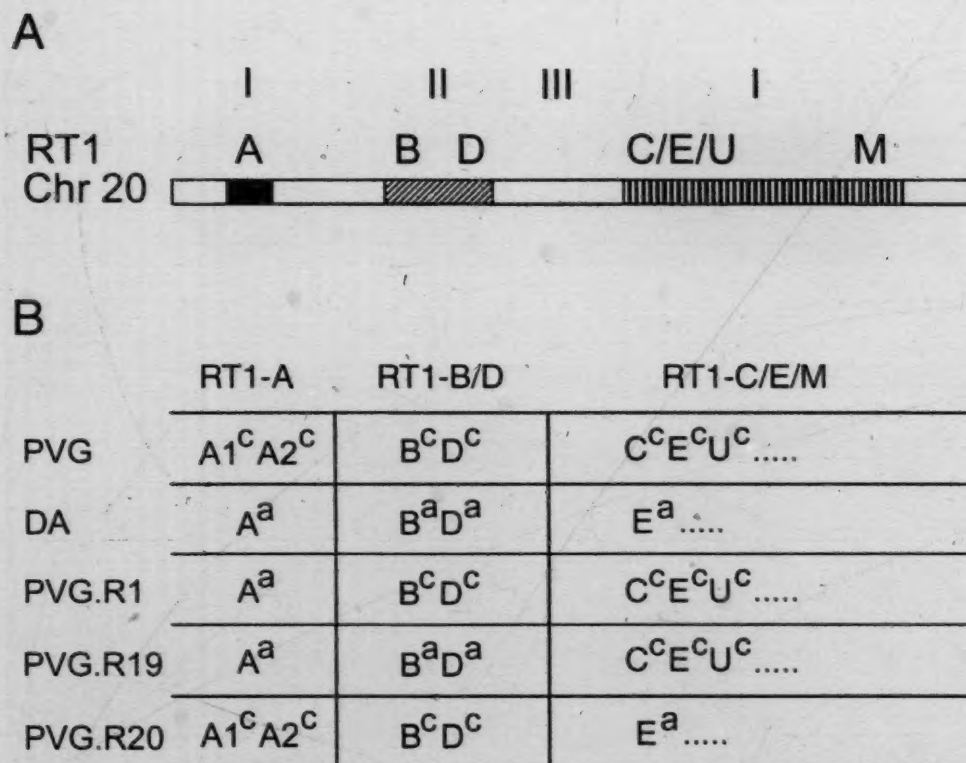


**Figure 2-2.** The rat major histocompatibility complex and haplotypes of selected rat strains. The RT1 complex is encoded on rat chromosome 20 and contains in order (centromeric to telomeric) the classical class I region RT1-A, the class II region RT1-B/D, the class III region and the RT1-C/E/M non-classical class I region, which encodes numerous molecules that are not fully characterized (A). The inbred rat strain, PVG, expresses MHC molecules of the RT1<sup>C</sup> haplotype while the DA strain expresses molecules of the RT1<sup>av</sup> haplotype. PVG congenic strains which have undergone recombination events in the RT1 region, express MHC molecules of either the RT1<sup>C</sup> or RT1<sup>av</sup> haplotype, depending on the location of the recombination event, as indicated (B).

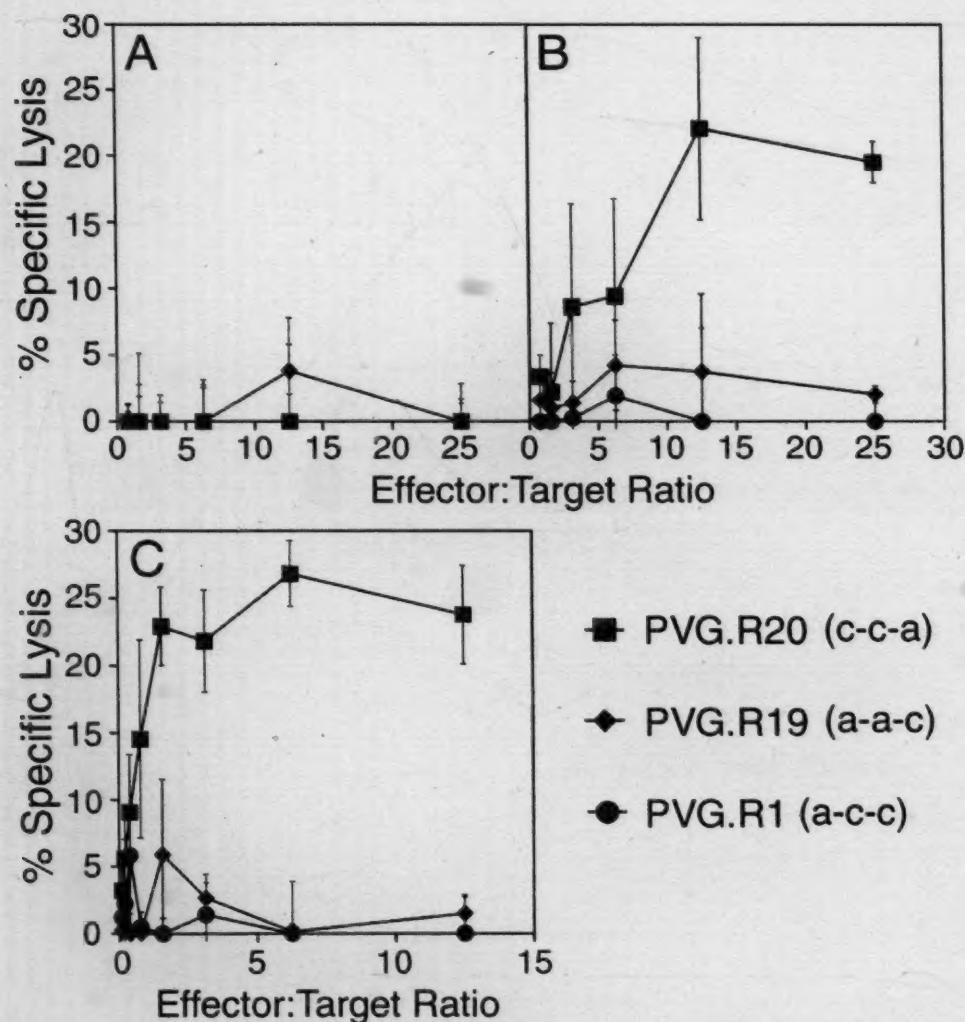


**Figure 2-3.** Lysis of RT1<sup>av1/c</sup> intra-MHC recombinant targets identifies the classical class I region of the RT1<sup>e</sup> haplotype as essential for recognition by Ly-49W and Ly-49WG<sup>BALB/c</sup>. RNK-16 cells (A), RNK-16 cells transfected with the activating Ly-49W receptor (B), or the chimeric activating receptor Ly-49WG<sup>BALB/c</sup> (C) were assayed for cytotoxicity against Con A blasts prepared from congenic, RT1<sup>av1/c</sup> intra-MHC recombinant rat strains in 4-h cytotoxicity assays. The PVG congenic, intra-MHC recombinant rat strains were PVG.R20 (c-c-a), PVG.R19 (a-a-c) and PVG.R1 (a-c-c). Data represent the mean of triplicate wells  $\pm$  SD.

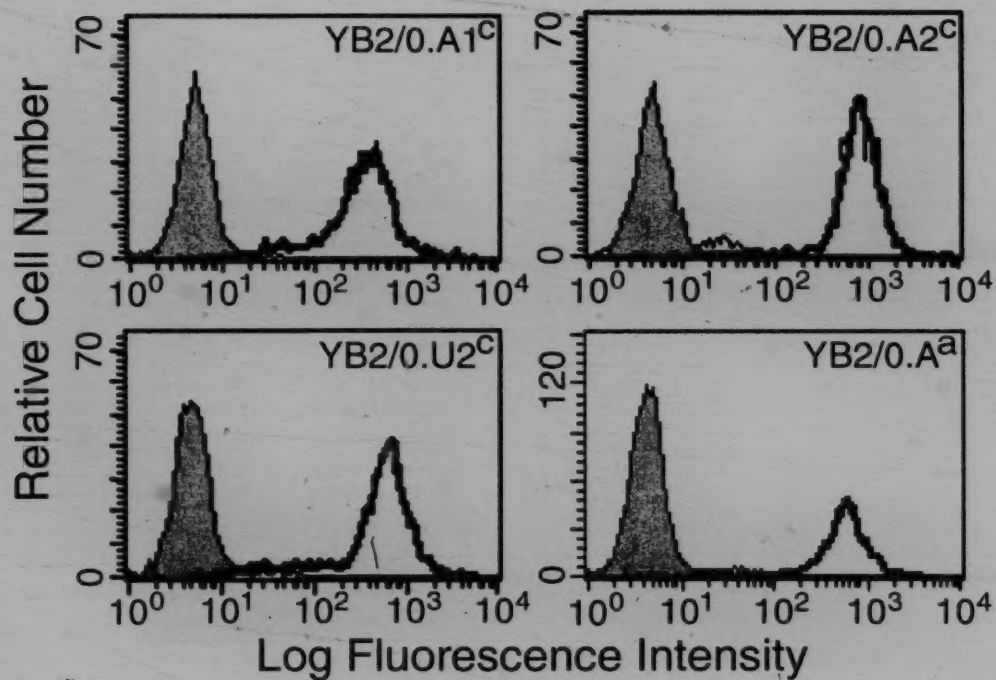


**Figure 2-4.** Expression of rat MHC-EGFP fusion proteins on transfected rat YB2/0 cells. The rat MHC molecules RT1-A1<sup>c</sup> (*upper left panel*), RT1-A2<sup>c</sup> (*upper right panel*), RT1.U2<sup>c</sup> (*lower left panel*) and RT1-A<sup>a</sup> (*lower right panel*) were C-terminally fused to EGFP for detection by flow cytometric analysis when transfected into the rat myeloma cell line, YB2/0. Transfected YB2/0 cells (*open histograms*) were compared for EGFP fluorescence relative to untransfected YB2/0 cells (*shaded histograms*).

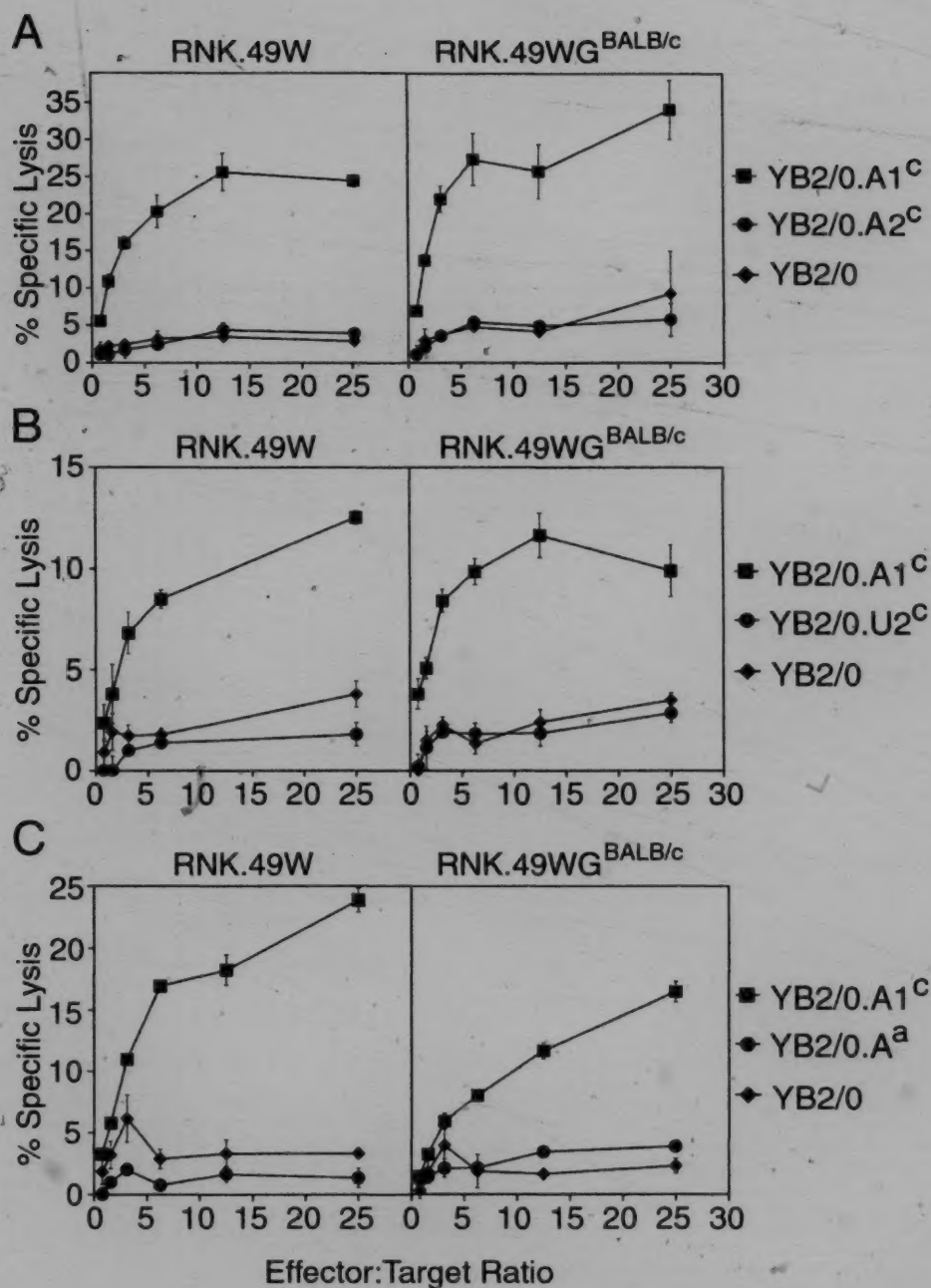


**Figure 2-5.** Ly-49W and the Ly-49WG<sup>BALB/c</sup> chimera recognize RT1-A1<sup>c</sup>, a classical class I MHC molecule from the PVG rat strain. RNK-16 cells transfected with Ly-49W or Ly-49WG<sup>BALB/c</sup> were examined for their ability to lyse YB2/0 cells and YB2/0 transfected with RT1-A1<sup>c</sup> compared with YB2/0 transfected with RT1-A2<sup>c</sup> (A), RT1-U2<sup>c</sup> (B) or RT1-A<sup>a</sup> (C). Data represent the mean of triplicate wells  $\pm$  SD.



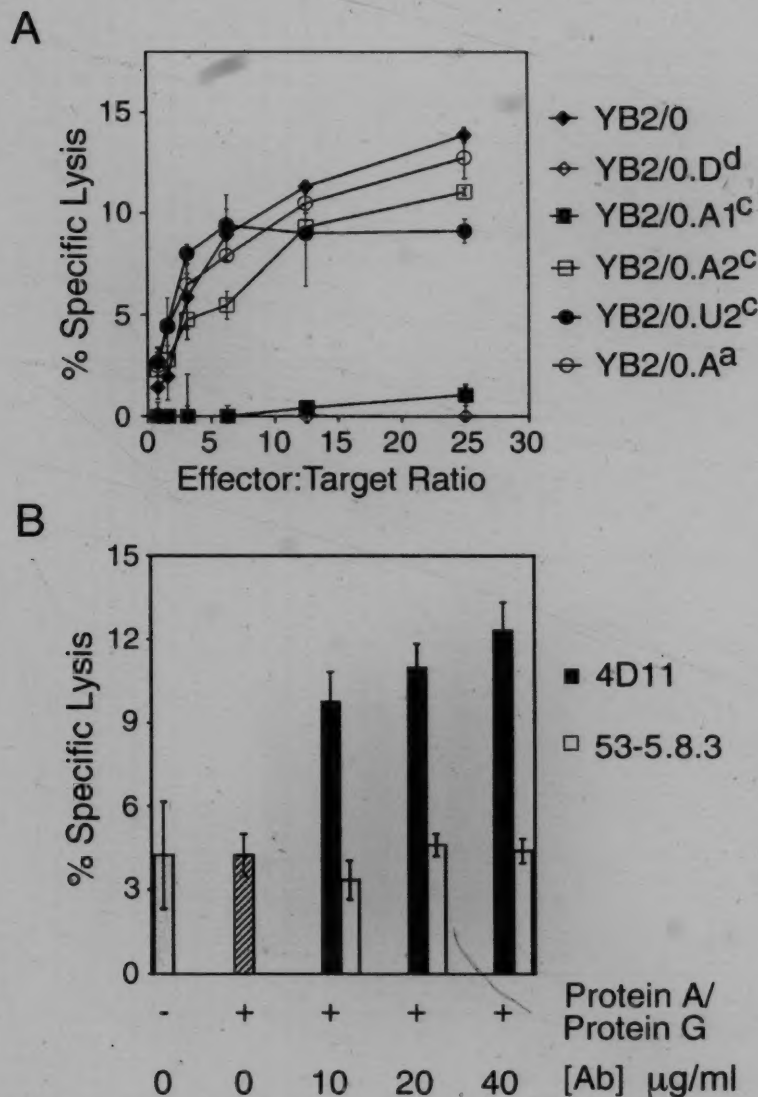


**Figure 2-6.** The inhibitory Ly-49G<sup>BALB/c</sup> receptor recognizes RT1-A1<sup>c</sup>. Ly-49G<sup>BALB/c</sup> was examined for its ability to inhibit cytolysis of YB2/0 cells or YB2/0 cells expressing RT1-A1<sup>c</sup>, RT1-A2<sup>c</sup> or RT1-U2<sup>c</sup> from the PVG rat strain or RT1-A<sup>a</sup> from the DA rat strain. RNK-16 cells transfected with Ly-49G<sup>BALB/c</sup> were incubated with YB2/0 cells or YB2/0 transfected with the indicated mouse or rat MHC molecules in a 4-h cytotoxicity assay (A). Ly-49G<sup>BALB/c</sup> transfected RNK-16 cells were incubated with YB2/0 RT1-A1<sup>c</sup> transfectants in the presence of 4D11 or 53-5.8.3 (isotype control) antibodies in a 4-h cytotoxicity assay. Protein A/G was included at a 1:1 ratio to prevent rADCC (B). Data represent the mean of triplicate wells  $\pm$  SD.

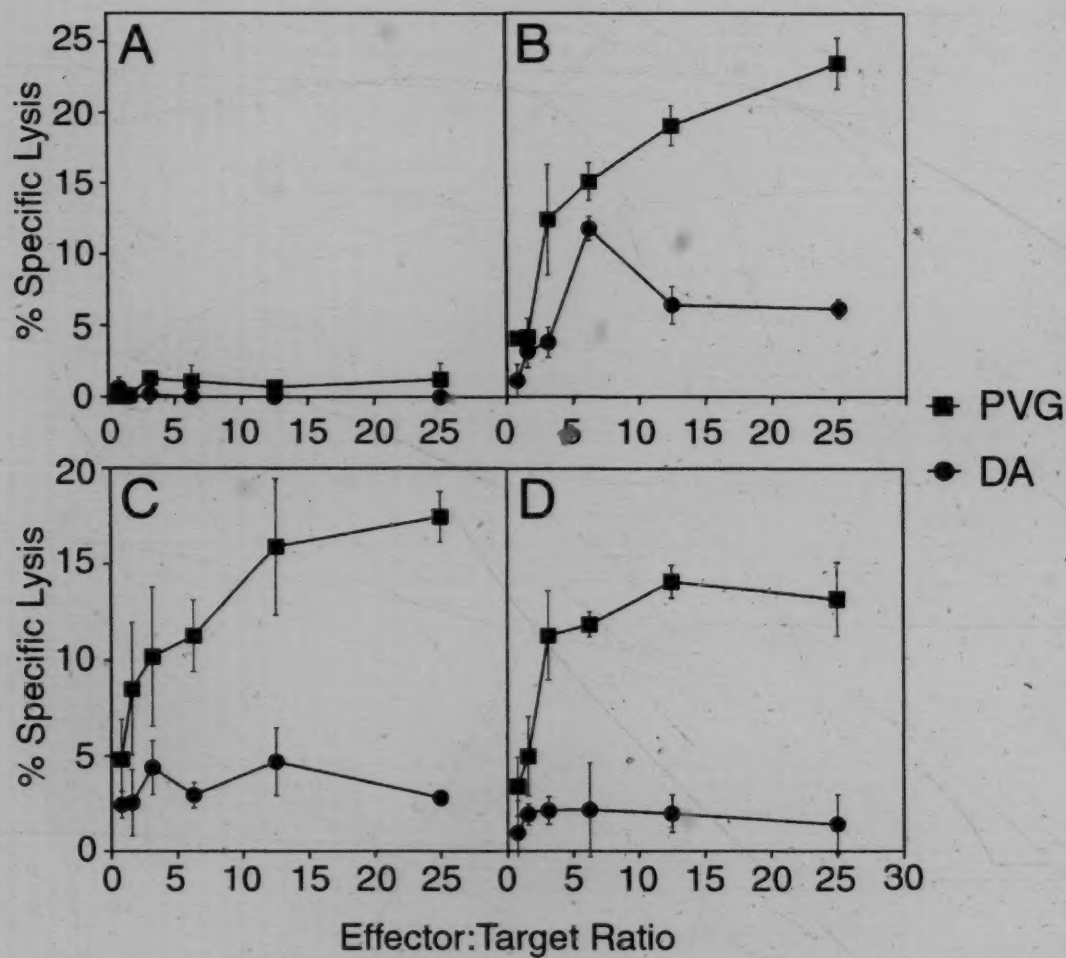


**Figure 2-7.** Mutagenesis of the Ly-49P  $\beta 4$ - $\beta 5$  loop to express the Ly-49W motif DCGK confers specificity for a ligand in the PVG rat strain. Recognition of Con A blasts from the PVG and DA rat strains by Ly-49P (A), mutant Ly-49P (B), Ly-49W (C) and mutant Ly-49W (D) were examined in 4-h cytotoxicity assays. Data represent the mean of triplicate wells  $\pm$  SD.

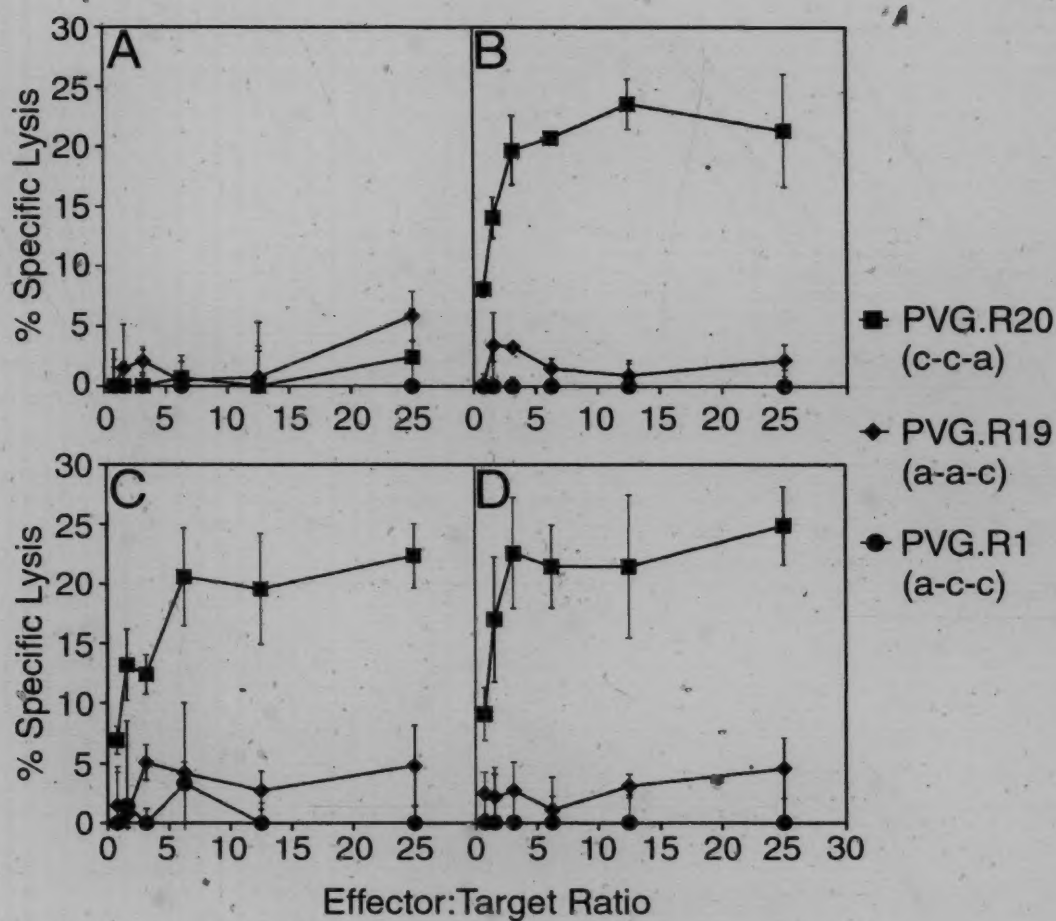


**Figure 2-8.** The Ly-49P and Ly-49W  $\beta 4$ - $\beta 5$  loop mutants recognize RT1-A<sup>e</sup> encoded ligand(s). Lysis of Con A blasts from the indicated PVG intra-MHC recombinants by Ly-49P (A), mutant Ly-49P (B), Ly-49W (C) or mutant Ly-49W (D) was determined in a 4-h cytotoxicity assay. Data represent the mean of triplicate wells  $\pm$  SD.

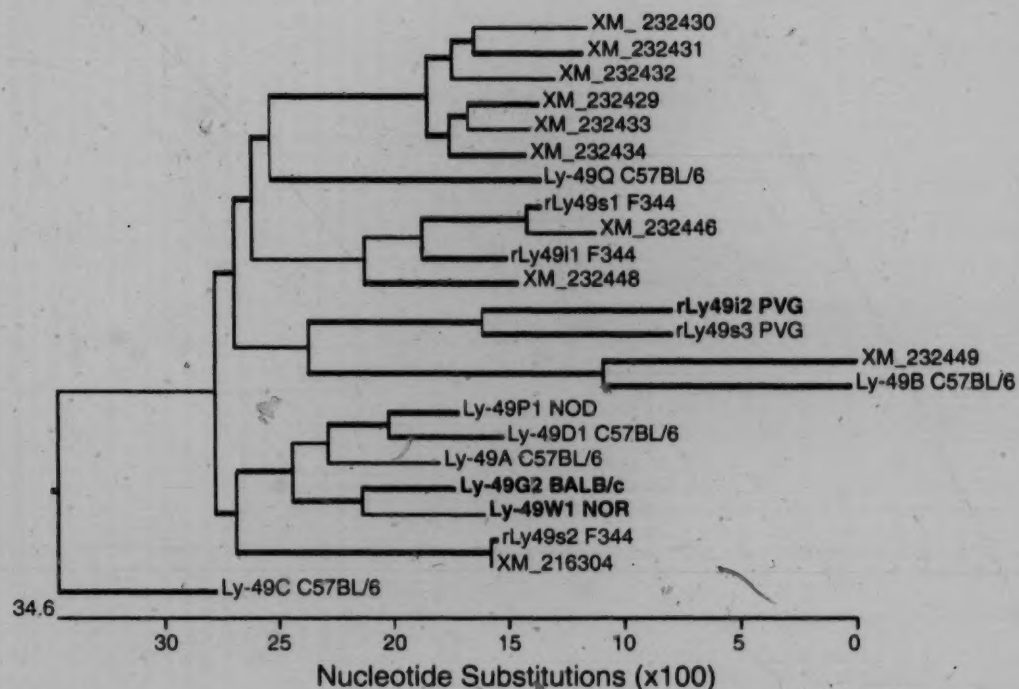


**Figure 2-9.** Dendrogram comparing the nucleotide sequences of five known and ten predicted rat Ly-49 receptors to eight mouse Ly-49 receptors. Ly-49 receptor sequences are available from GenBank: (Ly-49A, M25812; Ly-49B, AF253058; Ly-49C, U56404; Ly-49D, L78247; Ly-49G, AF307946; Ly-49P, AF218080; Ly-49Q, AB033769; Ly-49W, AF074459; Ly49i1, U56863; Ly49i2, NM\_152848; Ly49s1, U56822; Ly49s2, U56822; Ly-49s3, NM\_153726). Ten rat Ly-49 receptors, predicted through automated computational analysis of genomic sequence from the BN/SsNHsd/MCW rat strain (NW\_043770), are represented by their accession numbers. DNA sequences were aligned with CLUSTALW (69) and the dendrogram created in MegAlign (DNASTAR Inc., Madison WI).



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## CHAPTER III

### CROSS-SPECIES DEPENDENCE OF LY49 RECOGNITION ON THE SUPERTYPE DEFINING B-POCKET OF A CLASS I MHC MOLECULE<sup>1,2</sup>

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#### A. Introduction

Natural Killer (NK) cells are large granular lymphocytes that play an important role in innate immunity against transformed and virally infected cells through both cytolytic and cytokine mediated effector responses (1). Murine and human NK cells express a number of receptors on their cell surfaces, including Ly49 and killer cell immunoglobulin-like receptors (KIR), respectively. Although structurally distinct, both of these receptor types are polygenic and have activating and inhibitory members capable of recognizing MHC class I (MHC I) proteins (2, 3). Inhibitory receptors function according to the "missing self hypothesis", surveying potential target cells for expression of self MHC I molecules and transmit inhibitory signals that prevent NK cell activation and target cytotoxicity (4). In rodents, upon loss of MHC I expression, due to virus infection or cell transformation, inhibitory Ly49 receptors may not be engaged and lysis and cytokine production can occur through engagement of activating Ly49 or other activating NK cell receptors (5). In addition to MHC I, a virally encoded MHC I homolog was

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shown to be a ligand for both activating and inhibitory Ly49 receptors (6, 7).

Ly49 are MHC I allele specific, each having specificity for a small repertoire of MHC I alleles that are generally recognized in a peptide independent manner (8-15). What determines allele specificity of Ly49 receptors is not understood. The original co-crystal of Ly49A and its ligand H-2D<sup>d</sup> showed two potential interaction sites on the MHC I molecule (16) (Fig. 3-5A). Site 1 is located at one end of the polymorphic peptide-binding groove and was initially favored as the interaction site, conferring allele specificity for Ly49 recognition (16). Later, site 2 was shown to be the main interaction site through mutagenesis of single solvent exposed residues under the peptide-binding platform and in the alpha 3 domain of the MHC I heavy chain, as well as residues within  $\beta$ 2-microglobulin (17, 18). Since site 2 is so highly conserved between different MHC I alleles, it is difficult to explain allele specificity by Ly49 receptors at this recognition site.

Murine and primate (including human) MHC I alleles can be classified into about nine different supertypes based on their preference for specific amino acid residues within the bound peptide, which act to anchor it within the peptide-binding groove, in specific anchoring pockets (19). The equal distribution of supertypes across species and even human ethnicities emphasizes the importance of supertypes for the effective function of the immune system against invading pathogens (20-22). Previously we identified a xenogeneic rat MHC I ligand, RT1-A1<sup>c</sup>, for two mouse Ly49 receptors, the activating Ly49W and the BALB/c allele of the inhibitory Ly49G (23). The RT1-A1<sup>c</sup> molecule is also the natural ligand for the syngeneic inhibitory rat Ly49i2 receptor (24). We noticed that there was a common supertype between MHC I alleles that were recognized by these two mouse Ly49 receptors and the rat Ly49i2. Possibly then, the distinctive molecular characteristics associated with this supertype could be critical for recognition by these rodent Ly49 receptors.

Through single amino acid mutations of RT1-A1<sup>c</sup> we show here that both xenogeneic mouse and syngeneic rat Ly49 recognition are exquisitely sensitive to even conservative changes in polymorphic residues within the supertype defining B-pocket. In contrast, non-conservative single mutations within the peptide-binding groove, but outside of the B-pocket, have no effect. We also show that both rat syngeneic and mouse xenogeneic Ly49 recognition occurs at site 2, in a manner analogous to mouse syngeneic

Ly49 recognition. Crystal structure conformations of rodent MHC I alleles indicate that alterations in the B-pocket may result in changes in the conformation of solvent exposed residues at site 2, that articulate with the B-pocket, thereby affecting Ly49 recognition. That mouse Ly49 receptors recognize a MHC I supertype across species indicates that this may be a fundamental property of Ly49 interaction with MHC I that transcends species specific recognition, in that the supertype defining pocket(s) of MHC I offers identity to NK cells of innate immunity, while preserving conformations essential to MHC restriction for TCR interaction and adaptive immunity.

## **B. Materials And Methods**

### *Hybridomas and monoclonal antibodies*

The hybridoma producing the antibody 4D11 (rat IgG2a), anti-Ly49G<sup>BALB/c</sup> (9) was obtained from American Type Culture Collection (Manassas, USA). The Cwy-3 (IgG1), anti-Ly49W hybridoma was generated in this laboratory (25). Antibodies were prepared from ammonium sulfate precipitates as described (11). Purified STOK2 (rat IgG2a), anti-Ly49i2 (24, 26) antibody was purchased from BD Biosciences Pharmingen (San Diego, USA). The RT1-A1<sup>c</sup> reactive YR5/12 (rat IgG2b) hybridoma supernatant (27) was purchased from Serotec (Oxford, UK).

### *Cell lines*

YB2/0, a non-secreting rat myeloma, was obtained from American Type Culture Collection. The YB2/0 cell line was maintained in DMEM supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, 1mM sodium pyruvate and 0.1mM non-essential amino acids. RNK-16 is a spontaneous F344 rat strain NK cell leukemia cell line (28). RNK-16 cells were maintained in RPMI supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and  $5 \times 10^{-5}$ M 2-mercaptoethanol. RNK-16 effector cells expressing murine Ly49 receptors were generated by this lab as described (12, 29). All transfected RNK-16 and YB2/0 cells were maintained under G418 selection. Transfected cells were grown in the absence of G418 for at least 48 hours prior to cytotoxicity assays.



#### *Cloning and transfection of Ly49i2*

PVG rats were obtained from Harlan Sprague Dawley (Indianapolis, USA). Experiments were approved by the animal Welfare and Policy Committee of the University of Alberta (Edmonton, Canada). Total RNA was isolated from  $1 \times 10^7$  PVG rat lymphokine activated killer cells using an RNeasy Protect mini-kit (Qiagen, Valencia, USA). cDNA was produced using Powerscript reverse transcriptase (Clontech, Palo Alto, USA) with an oligo(dT) primer. Ly49i2 was amplified with Advantage-HF 2 polymerase mix using primers designed from the sequence NM\_152848 published by Naper et al. (24), digested with XhoI/XbaI and ligated into BSR $\alpha$ EN (Dr. A. Shaw, Washington University, St. Louis, USA). RNK-16 cells were transfected as previously described (12) and maintained under G418 selection until 48 hours prior to cytotoxicity assays.

#### *Mutagenesis of RT1-A1<sup>c</sup>*

RT1-A1<sup>c</sup>, previously cloned in this laboratory from PVG rat spleen (23), was mutated using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, USA). Mutagenic primers were designed to mutate polymorphic residues in the B-pocket of RT1-A1<sup>c</sup> (D9A, S24A, Y67A), residues within other pockets of the peptide-binding groove (F74A, F116A, F152A, D150A) and at site 1 (R169S) and site 2 (R6A, R111A) as defined by the Ly49A/H-2D<sup>d</sup> co-crystal (16). All mutations were verified by DNA sequencing. Mutagenesis was performed on RT1-A1<sup>c</sup> directly within the H-2D<sup>k</sup> leader-Enhanced GFP (EGFP) fusion vector that was previously generated in this lab (23), and which has been modified slightly to include a 6x histidine tag on the C-terminus of the MHC I-EGFP fusion protein. YB2/0 cells were stably transfected with each construct as previously described (11), and maintained under G418 selection until 48 hours prior to cytotoxicity assays.

#### *Flow cytometric analysis*

Successful transfection of class I MHC constructs was determined by EGFP fluorescence intensity of transfected YB2/0 cells relative to untransfected YB2/0 cells using a FACScan flow cytometer (BD Biosciences, Mountain View, USA). Proper folding and surface expression levels were determined using specific primary antibody to



RT1-A1<sup>e</sup> (YR5/12) plus R-Phycoerythrin-conjugated AffinPure F(ab')<sub>2</sub> Fragment Donkey Anti-Rat IgG secondary antibody (Jackson ImmunoResearch Laboratories Inc., Pennsylvania, USA). Expression of Ly49 receptors by RNK-16 cells was monitored compared to untransfected RNK-16 cells using primary antibodies 4D11 (anti-Ly49G<sup>BALB/c</sup>), Cwy-3 (anti-Ly49W) and STOK2 (anti-Ly49i2) followed by FITC-conjugated anti-rat or anti-mouse secondary antibody as required. All cells were incubated with normal mouse serum (for rat primary antibodies) or rat IgG (for mouse primary antibodies), to block Fc receptors prior to staining.

#### *Cytotoxicity assays*

Target cells were labeled with 100-150  $\mu$ Ci of Na<sup>51</sup>CrO<sub>4</sub> (Mandel, Guelph, Canada) at 37°C for 1 hour. Targets were washed three times with RPMI and 1x10<sup>4</sup> of Na<sup>51</sup>CrO<sub>4</sub> labeled cells were incubated with RNK-16 or transfected RNK-16 cells for 4 hours at 37°C in V-bottom microtitre plates at various E:T ratios in triplicate. After incubation, plates were centrifuged for 5 minutes at 1500rpm and 25  $\mu$ l of supernatant collected and counted in a MicroBeta TriLux liquid scintillation counter (PerkinElmer, Wellesley, USA). Percent specific lysis was determined as (experimental release – spontaneous release)/(maximal release – spontaneous release) x 100%.

#### *Peptide elutions*

Transfectants of YB2/0 expressing His-tagged wild-type or B pocket mutants of RT1-A1<sup>e</sup> were each grown in 20L in spinner culture flasks to a total of approximately 1x10<sup>10</sup> cells. Cells were centrifuged and washed in wash buffer (10mM Tris pH7.7 and 150mM NaCl) before being lysed at room temperature for 30 minutes in lysis buffer (wash buffer plus 1.5% NP-40 and 0.5mM PMSF). Lysate was centrifuged for 30 minutes at 20,000g at 4°C before adding 5mM imidazole plus 1.5ml Ni-NTA agarose beads (Qiagen, Valencia, USA) to the supernatant, with mixing, for 90 minutes at 4°C. Beads were then washed 5x with lysis buffer plus 20mM imidazole before one final wash with wash buffer. Peptides were then eluted using 10% glacial acetic acid for 5 minutes. Beads were removed using a 0.22  $\mu$ m low protein binding filter and MHC I heavy chain and  $\beta$ 2-microglobulin were removed using an Amicon Ultra filter device MW5000

(Millipore, Billerica, USA). Samples were diluted to 0.1M acetic acid and sent for Edman degradation at the Nucleic Acids and Protein Synthesis (NAPS) facility at the University of British Columbia (Vancouver, Canada) and the Advanced Protein Technology Centre Peptide Sequencing Facility at the Hospital for Sick Children (Toronto, Canada).

### C. Results

#### *RT1-A1<sup>c</sup> recognition by a syngeneic rat inhibitory receptor and xenogeneic mouse inhibitory and activating receptors expressed on RNK-16 cells*

Recognition of syngeneic MHC I molecules by mouse and rat Ly49 receptors can be MHC I allele specific (30, 31). Furthermore, as we and others have shown, mouse Ly49 can recognize xenogeneic MHC I molecules, and this too is MHC I allele specific (23, 29, 32). However, the role of polymorphic residues of MHC I molecules in determining allele specific recognition by Ly49 receptors is poorly understood. We previously identified allele specific xeno-reactivity of the mouse inhibitory Ly49G<sup>BALB/c</sup> and the activating Ly49W receptors for the PVG rat classical MHC I molecule RT1-A1<sup>c</sup> (23). From these findings, we set out to characterize the MHC I motifs that confer xeno-recognition, and to compare them to those required for syngeneic recognition of RT1-A1<sup>c</sup> by its natural syngeneic PVG rat strain receptor, Ly49i2 (24), which is also allele specific. In doing so, we aimed to obtain fundamental insights into Ly49 recognition of MHC I ligands. RNK-16 cells, which exhibit equal background cytotoxicity toward YB2/0 cells or YB2/0 cells expressing the rat MHC I molecule RT1-A1<sup>c</sup> (data not shown), were transfected with the mouse Ly49G<sup>BALB/c</sup> inhibitory receptor, the mouse Ly49W activating receptor, or the rat Ly49i2 inhibitory receptor (Fig. 3-1A). Recognition of RT1-A1<sup>c</sup> expressing YB2/0 cells by the inhibitory receptors Ly49G<sup>BALB/c</sup> and Ly49i2 is demonstrated by the nearly complete reduction in cytolysis by RNK-16 transfectants expressing these receptors, in comparison to normal YB2/0 cells (Fig. 3-1B). Recognition of RT1-A1<sup>c</sup> by the activating receptor Ly49W is observable through a substantial increase in cytolysis of YB2/0 cells expressing RT1-A1<sup>c</sup>, in comparison to the background of unaltered YB2/0 cells (Fig. 3-1B). Thus, the rat MHC I molecule, RT1-A1<sup>c</sup>, is recognized by inhibitory and activating xenogeneic mouse-Ly49 receptors and by the syngeneic Ly49i2 receptor as reported (23, 24).

*Xenogeneic mouse and syngeneic rat Ly49 recognition of RT1-A1<sup>c</sup> follows a pattern similar to mouse syngeneic recognition*

In order to characterize the MHC I motifs that confer xenogeneic Ly49 recognition, and to apply these findings to the identification of a motif that determines the allele specificity of Ly49 receptors, we needed to determine whether xenogeneic recognition occurs similarly to syngeneic recognition. The original co-crystal structure of Ly49A and H-2D<sup>d</sup> identified two different potential interaction sites on the MHC I molecule for recognition by Ly49A (16) (Fig. 3-5A). Site 1 is located at the end of the peptide-binding groove, is polymorphic among MHC I molecules and was initially a favorable candidate for mediating MHC I allelic specificity of Ly49 receptors. Site 2 is non-polymorphic and located under the peptide-binding platform, involving contact sites in all three domains of the heavy chain as well as residues within  $\beta$ 2-microglobulin. Mutagenesis of mouse MHC I residues has shown that site 2, and not site 1, is important for recognition by syngeneic mouse Ly49 receptors (17, 18). To determine whether rat syngeneic or mouse xenogeneic Ly49 recognition of RT1-A1<sup>c</sup> involved site 1, we mutated RT1-A1<sup>c</sup> at the previously identified site 1 residue, R169 (Fig. 3-5B) (16), and expressed this mutant to similar levels as wild-type RT1-A1<sup>c</sup> on YB2/0 cells (Fig. 3-2A). This residue was previously shown to affect recognition of H-2D<sup>d</sup>, by the activating receptor Ly49D, when mutated to the corresponding residue of the unrecognized H-2D<sup>b</sup> molecule (33). Therefore, we mutated this site 1 residue in RT1-A1<sup>c</sup> to a serine residue, which is found at this position in the non-recognized rat MHC I allele product, RT1-A<sup>a</sup> (34). None of the receptors, Ly49G<sup>BALB/c</sup>, Ly49W nor Ly49i2, appeared to mediate recognition of RT1-A1<sup>c</sup> through site 1, as the R169S mutant was recognized in a similar manner to the wild-type form of the MHC I molecule by all three receptors (Fig. 3-2B). This finding is in common with the recognition of H-2D<sup>d</sup> by Ly49A, which also is not affected by this site 1 mutation (17, 33).

To assess whether rat syngeneic and xenogeneic recognition occurs at site 2 we mutated RT1-A1<sup>c</sup> to alanine at two different residues, R6 and R111 (Fig. 3-5B), which are conserved in rat and mouse MHC I molecules. These residues were previously shown to be important for Ly49A recognition of H-2D<sup>d</sup> (16, 17). Both mutants were expressed on



YB2/0 cells at similar levels compared to the wild-type RT1-A1<sup>c</sup> (Fig. 3-2A). We found that xenogeneic recognition by Ly49G<sup>BALB/c</sup> and Ly49W followed a similar pattern to the previously demonstrated mouse syngeneic recognition of H-2D<sup>d</sup> by Ly49A (17) with R6A completely disrupting recognition and R111A having a partial effect (Fig. 3-2B). However, syngeneic recognition of RT1-A1<sup>c</sup> by Ly49i2 was not affected by the site 2, R6A or R111A mutations (Fig. 3-2B). Additionally, a double R6A R111A mutant of RT1-A1<sup>c</sup> did not result in any loss of recognition by the Ly49i2 receptor (data not shown). Residue K243, located in the alpha 3 domain of the MHC I molecule, has previously been shown to be important for Ly49A recognition of H-2D<sup>d</sup> (17) but did not disrupt recognition of RT1-A1<sup>c</sup> by Ly49i2 as a single mutant (data not shown). We then mutated RT1-A1<sup>c</sup> to create a R6A K243A double mutant and expressed it to similar levels as wild-type RT1-A1<sup>c</sup> on YB2/0 cells (Fig. 3-2C). The double site 2 mutant, R6A K243A, caused complete disruption of recognition by Ly49i2 (Fig. 3-2C). These results indicate that Ly49 recognition of RT1-A1<sup>c</sup> by the mouse Ly49G<sup>BALB/c</sup> inhibitory receptor, mouse Ly49W activating receptor and rat inhibitory Ly49i2 receptor, like syngeneic mouse recognition, is mediated through MHC I residues at site 2.

*Xenogeneic and syngeneic Ly49 recognition is sensitive to changes in B-pocket residues of RT1-A1<sup>c</sup>*

Since recognition of RT1-A1<sup>c</sup> by Ly49i2, Ly49G<sup>BALB/c</sup> and Ly49W was determined to be occurring at site 2 where there are no obvious polymorphic solvent exposed residues on the MHC I molecule that would confer allele specificity, we looked beyond amino acid sequence identity for a MHC I motif that could determine recognition of RT1-A1<sup>c</sup> by both syngeneic and xenogeneic Ly49 receptors. MHC I molecules bind peptides of about nine amino acids in length within the peptide-binding groove. Not all of the amino acids within the peptide play an equal role in positioning and stabilizing it within the folded peptide-MHC I complex, with two or three amino acids of the bound peptide acting as "anchor residues" within specific MHC I binding pockets. MHC I molecules can be grouped into supertypes based on their preference for defined peptide anchor residues bound within specific pockets of the MHC I peptide-binding cleft (19). We noted previously, that Ly49G<sup>BALB/c</sup> and Ly49W appear to recognize MHC I ligands of



a HLA-B7 supertype (23) that prefer to bind peptides with proline at the second, or P2 anchor position (H-2L<sup>d</sup> and RT1-A1<sup>c</sup>) or prefer a P2 residue with a small side chain, such as H-2D<sup>d</sup>, which uses P2 and P3 anchors residues of glycine and proline, respectively (35). Although only one MHC I ligand, RT1-A1<sup>c</sup> has been identified for Ly49i2 (24), it too may prefer MHC I molecules such as RT1-A1<sup>c</sup>, which bind small P2 residues. With this in mind, we examined the B-pocket of the MHC I molecule as its amino acid composition and three-dimensional structure determines the peptide residues that are capable of acting as P2 anchors. We first noted the presence of a large tyrosine residue at position 67 of RT1-A1<sup>c</sup> (Fig. 3-5B), which is not present in other non-recognized rat MHC I molecules, and could be responsible for the requirement of a small P2 residue to bind within the B-pocket. We mutated this residue to alanine and expressed the RT1-A1<sup>c</sup> Y67A mutant on YB2/0 target cells, matching its expression to that of YB2/0 cells expressing the wild-type RT1-A1<sup>c</sup> molecule (Fig. 3-3A). When we assayed for recognition of the Y67A mutant by the mouse Ly49G<sup>BALB/c</sup> and Ly49W receptors, we saw that alteration of this residue resulted in the loss of recognition by both xenogeneic receptors. In contrast, Ly49i2 recognition of the Y67A mutant was slightly reduced compared to wild-type RT1-A1<sup>c</sup> (Fig. 3-3B). We mutated two other B-pocket residues, D9 and S24 (Fig. 3-5B) to alanine residues and expressed these mutants on YB2/0 cells at similar levels to that of wild-type RT1-A1<sup>c</sup> (Fig. 3-3A). We used these additional mutants to determine if loss of recognition may be due to opening the B-pocket to larger P2 residues, as might occur with Y67A and D9A, or if recognition could be altered by a more conservative modification of the B-pocket, S24A, that likely would not change the preferred P2 residue. For all three receptors, recognition of the mutant RT1-A1<sup>c</sup> D9A was disrupted, with loss of recognition by the xenogeneic mouse receptors being complete and loss of recognition by the rat syngeneic receptor, Ly49i2, being only slightly less than complete (Fig. 3-3B). In a similar manner, YB2/0 cells expressing the RT1-A1<sup>c</sup> S24A mutant at comparable levels to wild-type RT1-A1<sup>c</sup> (Fig. 3-3A) also were not recognized by the inhibitory mouse receptor, Ly49G<sup>BALB/c</sup> nor the mouse activating receptor, Ly49W. Recognition of the S24A mutant by the rat inhibitory receptor, Ly49i2 was also lost, although again to a slightly lesser degree than the mouse receptors (Fig. 3-3B). Therefore, recognition of RT1-A1<sup>c</sup> by mouse xenogeneic and rat syngeneic receptors

is similar, in that recognition can be altered by mutations affecting single polymorphic amino acids within the B-pocket of RT1-A1<sup>c</sup>, including even a fairly conservative modification such as serine to alanine within the supertype defining B-pocket.

*Xenogeneic and syngeneic recognition of RT1-A1<sup>c</sup> is not sensitive to alterations of non B-pocket residues along the length of the peptide-binding groove*

Since xeno- and syngeneic Ly49 recognition of RT1-A1<sup>c</sup> was sensitive to alterations of residues in the B-pocket, we wanted to determine whether the receptors relied on the conformation of the B-pocket specifically or if their recognition could be altered by changes in other residues within the peptide-binding groove. We selected four different residues for mutagenesis to alanine: F74, F116, D150 and F152 (Fig. 3-5B). These residues were chosen for a number of reasons. Firstly, due to their positions on both the sides and bottom of the peptide-binding groove and secondly, their location within additional pockets of the peptide-binding groove. These residues were also chosen for their polymorphic nature, large size or the presence of charge, which would result in significant changes in groove structure upon mutagenesis to alanine. Each of these mutants were expressed on YB2/0 cells at similar expression levels as wild-type RT1-A1<sup>c</sup> (Fig. 3-4A) and assayed for recognition by the two mouse receptors, Ly49G<sup>BALB/c</sup> and Ly49W and rat Ly49i2 (Fig. 3-4B). Mutagenesis of F74, which faces into the groove on the same side as Y67 and lies just outside of the B-pocket, resulted in no loss of recognition by any of the three receptors (Fig. 3-4B), indicating no reliance on this portion of the peptide-binding groove for recognition by these receptors. Altering F116, a residue that lies in the center of the floor of the groove underneath residues P6 to P8 of the bound peptide, also did not result in any loss of recognition by either of the xenogeneic Ly49 receptors or the rat Ly49i2 receptor (Fig. 3-4B). Similarly, mutagenesis of D150, which lies on the opposite side of the groove from Y67 but more toward the C-terminal end of the bound peptide, resulted in no loss of recognition by any of the three Ly49 receptors tested (Fig. 3-4B). Lastly, mutagenesis of F152, which lies nearly opposite to F74 but more toward the center of the groove, also resulted in no loss of recognition by the mouse inhibitory Ly49G<sup>BALB/c</sup> receptor, the mouse activating Ly49W receptor, and the rat Ly49i2 receptor (Fig. 3-4B). Hence, both syngeneic and xenogeneic

recognition of RT1-A1<sup>c</sup> is not affected by the alteration of single residues within additional pockets of the peptide-binding groove, outside of the B-pocket.

*B-pocket mutants that disrupt syngeneic recognition are loaded with fewer peptides having proline at P2 while xenogeneic recognition can be disrupted without a change in P2 preference*

By altering residues within the B-pocket of RT1-A1<sup>c</sup>, we were potentially changing the size, charge and conformation of the pocket such that it no longer preferentially bound proline as its P2 anchor residue (36). Since Ly49C recognition of H-2K<sup>b</sup> has been shown to be dependent on the binding of specific peptides to H-2K<sup>b</sup> (37), potentially our RT1-A1<sup>c</sup> mutants were altering the B-pocket in such a manner that peptides with a different P2 anchor residue were binding and altering the RT1-A1<sup>c</sup> conformation and disrupting Ly49 recognition. To investigate this possibility we eluted peptides from wild-type RT1-A1<sup>c</sup> molecules and each of the B-pocket mutants expressed in the YB2/0 cell line. The yield and frequency of specific amino acids at defined positions within peptides, eluted in bulk, were determined by N-terminal Edman degradation sequencing and were compared to known elution profiles from PVG rat strain splenocytes (36, 38), which express this allele product naturally.

The sequencing we obtained from wild-type RT1-A1<sup>c</sup> expressed on YB2/0 cells agreed well with published results, showing a significantly higher molar yield of proline at P2 compared to any other residue (Table 3-1). Despite the change of a bulky tyrosine residue to a small alanine residue, which could hypothetically open the B-pocket to larger P2 residues, we still saw proline as the dominant P2 anchor residue being present in nearly half of peptides eluted from the Y67A mutant, which was able to disrupt xenogeneic recognition only (Table 3-1). The RT1-A1<sup>c</sup> D9A mutant potentially removed a charge constraint on which residues could act as P2 anchors of peptides that bound this RT1-A1<sup>c</sup> mutant. In this case, we continued to see proline as the dominant P2 anchor residue although its yield was reduced in comparison to wild-type and the Y67A mutant (Table 3-1) and the P2 yield of valine, a previously reported secondary RT1-A1<sup>c</sup> P2 anchor and HLA-B7 supertype P2 residue (35, 36, 38), was increased to nearly 25%. Similarly, peptides eluted from RT1-A1<sup>c</sup> S24A, a mutant that we did not expect to see



any alteration in the P2 residue, as the mutation is quite conservative, also continued to show proline as a dominant anchor residue at the P2 position (Table 3-1). Glutamine also appeared as a co-dominant anchor residue at P2 in peptides eluted from the S24A mutant (Table 3-1). Glutamine is not a previously reported P2 anchor residue for RT1-A1<sup>c</sup> nor an accepted HLA-B7 supertype P2 residue (19, 35). This may indicate that the S24A mutant, while maintaining a B7-supertype anchor profile in approximately 25% of loaded peptides, also bound in similar proportion to peptides with a specific non-B7 anchor residue, possibly contributing to the loss of Ly49 recognition of this mutant through an overall reduction of RT1-A1<sup>c</sup> molecules loaded with peptide of the B7-supertype.

Together, the peptide sequencing results suggest that the single B-pocket residue mutant Y67A, introduced in RT1-A1<sup>c</sup> through mutagenesis, could affect xenogeneic Ly49 recognition without a change in the dominant P2 anchor residue of bound peptides. In contrast, syngeneic recognition was disrupted only by B-pocket mutants that showed a reduced yield of proline at P2, suggesting that disruption of syngeneic recognition may require the increased loading of peptides without proline as a P2 anchor residue. Our results indicate that recognition of the RT1-A1<sup>c</sup> molecule by syngeneic and xenogeneic Ly49 receptors is dependent on, and extremely sensitive to, alterations in MHC I amino acids that specifically contribute to the composition of the polymorphic, peptide anchor residue binding, and supertype defining, B-pocket. These findings indicate that murine NK cells may play an important role in sensing either alterations in or loss of MHC I of a specific supertype.

#### **D. Discussion**

Ly49 receptors generally recognize MHC I molecules in an allele specific manner (30, 31), yet Matsumoto et al. (17) showed that the highly conserved site 2 region, which lies beneath the peptide-binding groove and includes residues in all three domains of the MHC I heavy chain and  $\beta$ 2-microglobulin (Fig. 3-5A) was the major site of interaction for Ly49 with MHC I molecules. This raises two questions, how do Ly49 receptors distinguish between polymorphic MHC I molecules and how has there been expansion of the Ly49 locus when Ly49 receptors interact with such a highly conserved region on MHC I molecules? By identifying RT1-A1<sup>c</sup> as a xenogeneic rat MHC I ligand for the



mouse receptors Ly49G and Ly49W (23), we thought that a polymorphic epitope that was conserved between species but differed between MHC I alleles might be apparent to explain the discrimination between rodent MHC I alleles by these receptors. In examination of site 2 of RT1-A1<sup>c</sup>, with known ligands and non-ligands for Ly49G and W we found that between the rat and mouse only two polymorphisms occur at site 2: Pro2 (H-2D<sup>k</sup>, D<sup>b</sup>, L<sup>d</sup>, K<sup>b</sup>) or Ser2 (H-2D<sup>d</sup>, RT1-A1<sup>c</sup>, A<sup>a</sup>) and Met138 (H-2D<sup>d</sup>, D<sup>k</sup>, D<sup>b</sup>, L<sup>d</sup>, K<sup>b</sup>) or Phe138 (RT1-A1<sup>c</sup>, A<sup>a</sup>). The polymorphisms at position 2 and at 138 do not differentiate Ly49G or W ligands from non-ligands, since Ly49G recognizes H-2D<sup>d</sup>, D<sup>k</sup>, L<sup>d</sup> and RT1-A1<sup>c</sup>, Ly49W recognizes only H-2D<sup>d</sup>, D<sup>k</sup> and RT1-A1<sup>c</sup>, and neither receptor recognizes H-2D<sup>b</sup>, K<sup>b</sup> or RT1-A<sup>a</sup>. Thus, it was not apparent how these polymorphisms could explain the allele specific recognition patterns observed.

What is in common between the MHC I alleles recognized by Ly49G, W and i2 is a similarity of supertype. MHC I molecules of numerous species are classified into about nine different superotypes based on the peptide residues that act to "anchor" the peptide into the MHC I molecule's peptide-binding groove (19). Most MHC I molecules tightly bind the second (P2) and last residues (P9) of the peptide in the anchor binding B- and F-pockets of the MHC I molecule, respectively, although other anchor/pocket combinations can occur. H-2L<sup>d</sup> fits perfectly into what is known as the HLA-B7 supertype, binding Pro as P2 in the B-pocket and Leu or Phe as P9 in the F-pocket of the MHC I molecule (35). Interestingly, we noted that the xenogeneic ligand, RT1-A1<sup>c</sup>, also fits into the HLA-B7 supertype (36) and although H-2D<sup>d</sup> is not a perfect example, it also best fits into the B7-supertype, binding small residues (Gly and Pro) in its anchoring B and C pockets, respectively (35). In contrast, non-recognized alleles did not fit the HLA-B7 supertype, and varied from it considerably. The H-2D<sup>b</sup> and K<sup>b</sup> molecules use the P5 residue to anchor peptides and prefer Asn and Phe/Tyr peptide anchors, respectively. RT1-A<sup>a</sup> also does not fit the HLA-B7 supertype and although it uses the B-pocket to anchor peptides it binds larger amino acids such as Leu, Gln and Met as preferred anchor residues (35). This led us to examine whether similarities in the MHC I B-pocket, which are shared between species but differ between MHC I alleles, could be the means by which Ly49 receptors differentiate between MHC I alleles. Indeed, upon examination of site 2 residues that lie beneath and articulate with the B-pocket of recognized alleles (RT1-A1<sup>c</sup>

and H-2D<sup>d</sup>) with those of non-recognized alleles (RT1-A<sup>a</sup>, H-2D<sup>b</sup>, H-2K<sup>b</sup>) it is apparent that solvent exposed side chains available for interaction at site 2 have an essentially identical conformational structure and position in RT1-A1<sup>c</sup> and H-2D<sup>d</sup> and a completely different conformational structure and position in RT1-A<sup>a</sup>, H-2D<sup>b</sup> and K<sup>b</sup> (Fig. 3-5C). Also, H-2L<sup>d</sup>, which is recognized weakly by Ly49G (29) and not by Ly49W (data not shown), has a similar but not identical conformational structure of the solvent exposed residues below the B-pocket, compared to RT1-A1<sup>c</sup> and H-2D<sup>d</sup>, that may explain its less than optimal interaction with Ly49G and W (Fig. 3-5C).

By modifying the site 2 residues R6 and R111 (Fig. 3-5B) we demonstrated that xenogeneic recognition of RT1-A1<sup>c</sup> follows an identical pattern of recognition to the mouse Ly49A/H-2D<sup>d</sup> interaction. Additionally, these site 2 residues lie beneath and articulate with the B-pocket, particularly R6, which when mutated, results in greater disruption of recognition compared to R111, which is less influenced by the B-pocket. This relationship supports the hypothesis that the side chain conformations of these and other solvent exposed residues that articulate with the B-pocket may become more or less available for optimal interaction with Ly49 receptors depending on B-pocket conformation (Fig. 3-5C). Ly49i2, like Ly49G and W, did not appear to be reliant on site 1 for recognition of RT1-A1<sup>c</sup>, but also did not show a similar sensitivity to single mutations of R6 and R111 at site 2. However, Ly49i2 does recognize RT1-A1<sup>c</sup> at site 2, as a double mutant including the B-pocket associated R6 residue and the previously identified site 2 residue K243 (Fig. 3-5B) of the class I molecule disrupted recognition, whereas single R6 or K243 (data not shown) mutants had no effect. This slightly altered dependence on site 2 residues by Ly49i2 may be expected, as Ly49i2 differs from Ly49G and W in two important ways that may alter how it interacts with residues below the B-pocket. First, Ly49i2 does not share the glycosylation motif NTT (221-223) that Ly49G and W possess, which is directly adjacent to the interaction site under the B-pocket at site 2 and might lessen the binding strength (39). Second, there is sequence dissimilarity between Ly49i2 and Ly49G/W (12, 24) in a region that could alter precisely how this receptor interacts with residues below the B-pocket. Therefore, Ly49i2 is more likely either interacting with greater affinity than Ly49G and W or has a greater reliance on different B-pocket associated solvent exposed residues. Nevertheless, the dependence on

B-pocket residues for Ly49i2 recognition is shared with the mouse Ly49G and W receptors.

In support of our hypothesis that both xenogeneic and syngeneic rat recognition was occurring at site 2 through solvent exposed residues that were influenced by B-pocket conformations, we found that both xenogeneic recognition by Ly49G and W and syngeneic recognition by Ly49i2 were very sensitive to changes within the B-pocket. By mutating polymorphic residues within the B-pocket of RT1-A1<sup>c</sup> to alanine we were able to show that Ly49 receptors could sense changes in floor conformation with the S24A mutant, changes in the charge of the pocket with the D9A mutant, and even showed xenogeneic sensitivity to structural changes higher up in the pocket with the Y67A mutant (Fig. 3-5B). While it is apparent that mutations in the floor of the B-pocket might directly influence the conformation of solvent exposed residues used in Ly49 recognition, it is less apparent how a mutation higher in the pocket could disrupt recognition. However, it has been previously suggested that "void" regions in proteins play a role in stability and function by affecting molecular packing in the protein interior (40). The Y67A mutant may affect the molecular packing of the B-pocket in this manner, translating to the solvent exposed interaction residues and disrupting the xenogeneic interaction at site 2. Mutations in other pockets within the peptide-binding groove of RT1-A1<sup>c</sup> changing the size (F74A, F152A) and charge (D150A) of pockets within the groove and the conformation of the groove platform (F116A) (Fig. 3-5B) did not alter recognition, showing a specific reliance on the supertype defining B-pocket and its potential effects on the conformation of solvent exposed residues that articulate with it. In examination of earlier work that mutated residues within the peptide-binding groove of H-2D<sup>d</sup>, but not specifically focusing on anchor binding pockets, we noticed that single mutants outside of the main anchor binding pockets were also unable to disrupt recognition by Ly49A and it required double and triple mutants close to or within minor anchor binding pockets to cause partial or complete disruption, respectively (33, 41). In contrast, we demonstrate here that single mutants within the supertype defining B-pocket of RT1-A1<sup>c</sup>, that do not prevent the MHC I molecule from binding peptide, had significant effects on both xenogeneic mouse and syngeneic rat Ly49 recognition.



Peptides eluted from the B-pocket mutants of RT1-A1<sup>c</sup> showed that disruption of xenorecognition and syngeneic rat Ly49 recognition through mutagenesis of the anchor binding and supertype defining B-pocket may occur through two different mechanisms. First, the Y67A mutant showed little alteration in proline yield as the P2 anchor, which is surprising, as this mutant would potentially open the B-pocket to larger P2 residues. This may be due to a reliance on the overall composition of the B-pocket, or residues deeper in the pocket, for alteration of P2 anchor binding preference. Since the Y67A mutant showed little or no alteration in P2 binding preference, likely the conformational change induced in the MHC I heavy chain through the Y67A mutation is the mechanism capable of disrupting xenogeneic but not syngeneic Ly49 recognition of this mutant. Second, although none of the mutants showed an overt change of supertype by complete displacement of proline as a dominant anchor, it is interesting to note that peptides eluted from the S24A and D9A mutants did show a lesser yield of proline at P2 of bound peptides. The S24A mutant is particularly interesting. The reduction in loaded peptides with proline at P2 was balanced by an increase of peptides containing glutamine at P2, a residue that is not recognized as either a P2 anchor for RT1-A1<sup>c</sup> (35) nor as a B7<sup>-</sup> supertype anchor residue (19). This may indicate that the loss of recognition of this mutant was due to the loss of a recognizable supertype, at sufficient density at the cell surface, for recognition by both syngeneic and xenogeneic Ly49 receptors. With D9A, the reduction in proline at P2 was balanced by an increase of valine, which is accepted as a secondary RT1-A1<sup>c</sup> (36) and HLA-B7 supertype anchor residue (19). This suggests that Ly49 recognition of MHC I may also be sensitive to alterations in the overall proportion of MHC I loaded with specific anchor residues without requiring an overt change in supertype. Therefore, in addition to direct conformational changes in the MHC I heavy chain, induced through B-pocket mutagenesis, the overall reduction of RT1-A1<sup>c</sup> molecules at the cell surface, loaded with peptides with a proline P2 anchor, could be an additional mechanism contributing to the loss of recognition of these mutants by Ly49.

Orihuela et al. demonstrated that Ly49A is not peptide selective; loading H-2D<sup>d</sup> with various peptides including a "skeletal" Ala peptide did not disrupt recognition (14). This peptide still required the presence of H-2D<sup>d</sup> anchor residues (AGPAAAAAL) to allow proper folding and presentation at the cell surface, but in the context of our present



study, may also have preserved a recognizable MHC I supertype associated conformation for Ly49A recognition. It is possible then that the reduction in proline at P2, and therefore the increased presence of MHC I loaded with peptides having either secondary P2 anchors or P2 anchors of a different supertype, was required for the loss of syngeneic but not xenogeneic Ly49 recognition we observed. An altered repertoire of bound peptides has been proposed to explain the gain of recognition of H-2D<sup>k</sup> by Ly49P upon MCMV infection (42). Should this be the case, it might be due to changing the structure of the MHC I molecule through altered dominance of different amino acids as P2 residues, thereby affecting recognition by Ly49 receptors.

KIR receptors also survey for changes in the peptide-binding preferences of the MHC I molecule (43) and this may be supertype related as KIR-3DL2 recognizes more than one MHC I allele of the HLA-A3 supertype when loaded with virus derived peptides, but not self peptides or MHC I of other superotypes (44). While early work demonstrated human NK receptor (likely KIR) sensitivity to B-pocket mutations of HLA-B7 (45), it is now clear that KIR interact with HLA nearer to the other anchor residue binding and supertype defining F-pocket (46). This suggests that, similar to what we have demonstrated with Ly49 recognition, some KIR may also survey for MHC I of specific superotypes. It is conceivable that KIR may be sensitive to small differences in the conformation of the F-pocket region of different MHC I superotypes in combination with different peptides, as opposed to the B-pocket sensitivity demonstrated here with rodent Ly49 receptors. KIR can also recognize xenogeneic MHC I ligands (47), and perhaps this is mediated through common supertype conformations at anchor binding pockets.

Why would Ly49 receptors survey for MHC I superotypes? The equal distribution of superotypes across species (20, 21) and even human ethnicities, although allele representation within those superotypes varies (48), implies that a certain complement of superotypes is required within a population to provide immune fitness toward a battery of pathogens (49). Since there are no MHC I orthologs between rat and mouse or between rodents and humans (50), and since examination of the B-pocket structure of murine vs. primate superotypes shows a large difference in the pocket structures, it appears that convergent evolution has occurred to produce similar superotypes in both mammalian orders. Superotypes likely are important for T cell recognition. It has been shown that the

TCR can be affected by more than the solvent exposed regions of MHC I, and changes in anchor binding pockets affect the structure and conformation of the MHC I surface that interacts with the TCR, altering recognition (51, 52). Therefore, MHC I allele specific inhibitory Ly49 receptors, such as Ly49A, G and i2 may be sensing whether specific MHC I supertypes are expressed to present peptides with specific anchor residues that would normally be presented to T cells. Loss of relevant supertype interaction with Ly49 inhibitory receptors, for example, would indicate that a cell may be incapable of presenting a major subset of peptides (with specific P2 anchors) to T cells and should be eliminated. In contrast, receptors such as Ly49C that recognized a broader repertoire of MHC I alleles (10), may be surveying more for the presence of properly folded MHC I capable of interacting with  $\beta$ 2-microglobulin and the CD8 T-cell co-stimulatory molecule, which shares portions of the Ly49 binding site on MHC I (53).

We demonstrate that some Ly49 are able to detect a supertype, even across species, and are sensitive to alterations specifically in the supertype-defining B-pocket. Therefore, we conclude that MHC I allele specificity of Ly49 receptors can be dictated by conformational differences between MHC I supertypes. Importantly, this would allow NK cells to survey for the presence or absence of MHC I supertypes capable of presenting peptide antigens to T cells, and elimination of cells defective in antigen presentation through loss of or alterations in a specific MHC I supertype. Such defects would result in the inability to present immunodominant peptides of a specific supertype or possibly loss of conformations important for MHC restriction. This shared dependence on MHC I supertypes by Ly49 and TCR indicates yet another fundamental link between innate and adaptive immunity. It also provides a potential explanation for the expansion of the Ly49 locus, through pressure to maintain Ly49 capable of detecting conformational differences in the MHC I supertypes that are essential to adaptive immunity.

#### **E. Acknowledgments**

We thank Dong-Er Gong for excellent technical assistance.

#### **F. Author Contribution to Data**

Kerry Lavender generated all the data produced in this study.

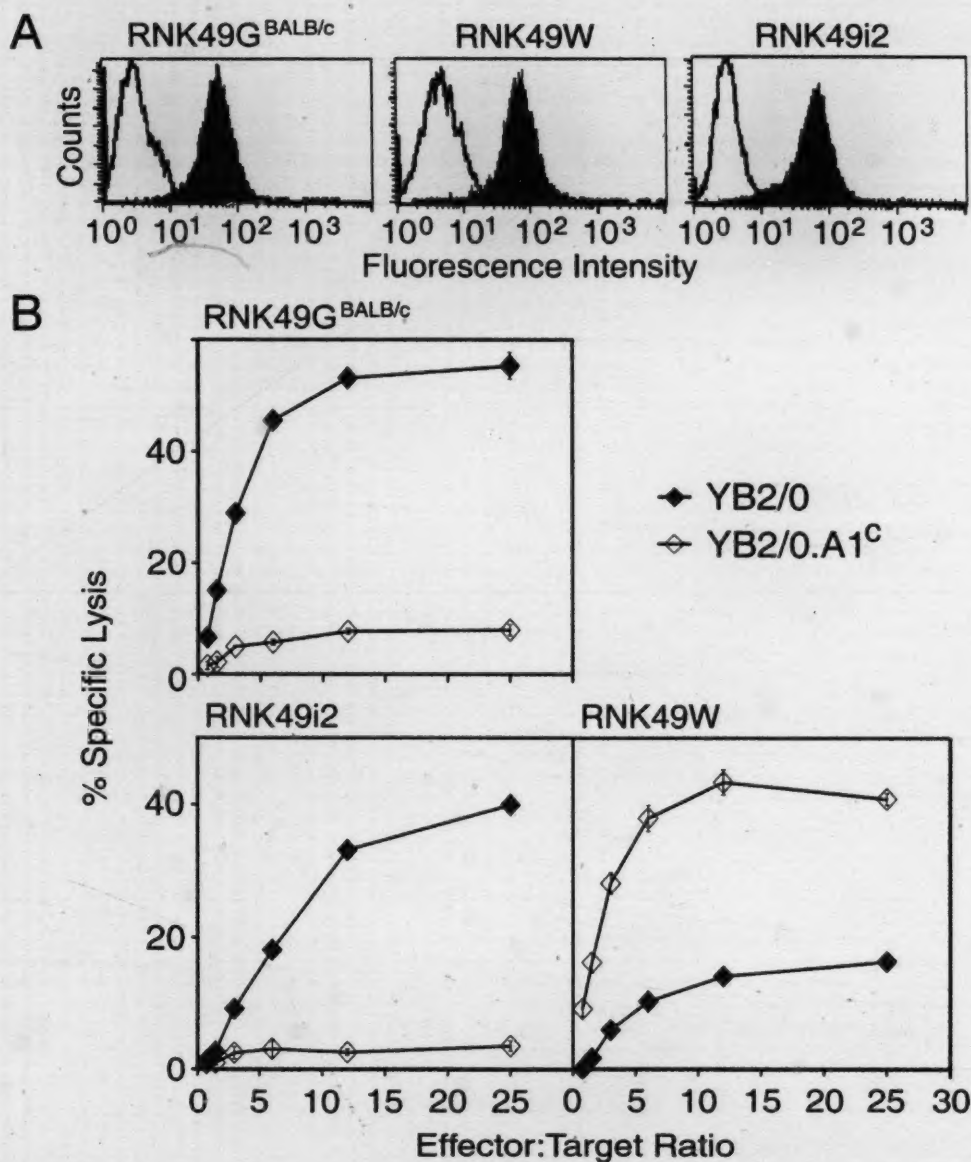


**Figure 3-1.** The rat classical class I MHC molecule, RT1-A1<sup>c</sup>, is a xenogeneic ligand for mouse Ly49G<sup>BALB/c</sup> and Ly49W and a syngeneic ligand for Ly49i2. Ly49G<sup>BALB/c</sup>, Ly49W and Ly49i2 were each expressed to similar levels on RNK-16 cells. *A*, Transfected RNK-16 cells (*shaded histograms*) were assessed for Ly49 specific antibody staining (4D11, Ly49G<sup>BALB/c</sup>; Cwy-3, Ly49W; STOK2, Ly49i2) compared to untransfected RNK-16 cells (*open histograms*). *B*, RNK-16 cells transfected with Ly49G<sup>BALB/c</sup>, Ly49W or Ly49i2 were compared for cytotoxicity against YB2/0 cells and YB2/0 cells transfected with RT1-A1<sup>c</sup>. Data represent the mean of triplicate wells  $\pm$  SD and are representative of three independent experiments.



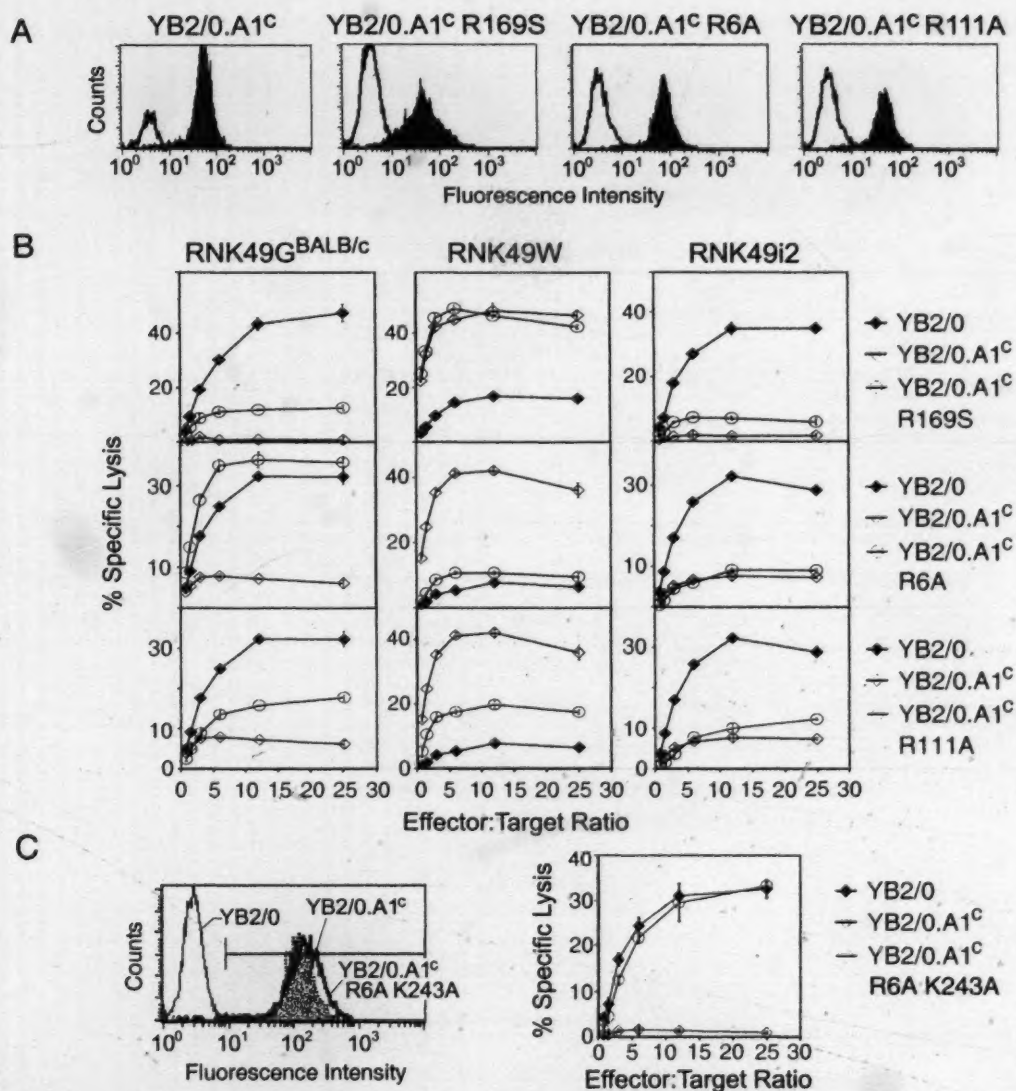


**Figure 3-2.** Xenogeneic and syngeneic Ly49 recognition of RT1-A1<sup>c</sup> is at site 2. *A*, Wild-type RT1-A1<sup>c</sup>, the site 1 mutant R169S and the site 2 mutants R6A and R111A (*shaded histograms*) were each expressed to similar levels on YB2/0 cells compared to untransfected YB2/0 cells (*open histograms*). *B*, RNK-16 cells transfected with Ly49G<sup>BALB/c</sup>, Ly49W or Ly49i2 were compared for cytotoxicity against YB2/0 cells and YB2/0 transfected with the RT1-A1<sup>c</sup> site 1 R169A mutant, site 2 R6A or R111A mutants or wild-type RT1-A1<sup>c</sup>. *C*, The site 2 RT1-A1<sup>c</sup> double mutant, R6A K243A (*thick lined histogram*), was expressed to similar levels as wild-type RT1-A1<sup>c</sup> (*shaded histogram*) compared with untransfected YB2/0 cells (*thin lined histogram*) and was assayed for cytotoxicity by RNK-16 cells transfected with Ly49i2. Data represent the mean of triplicate wells  $\pm$  SD and are representative of three independent experiments.

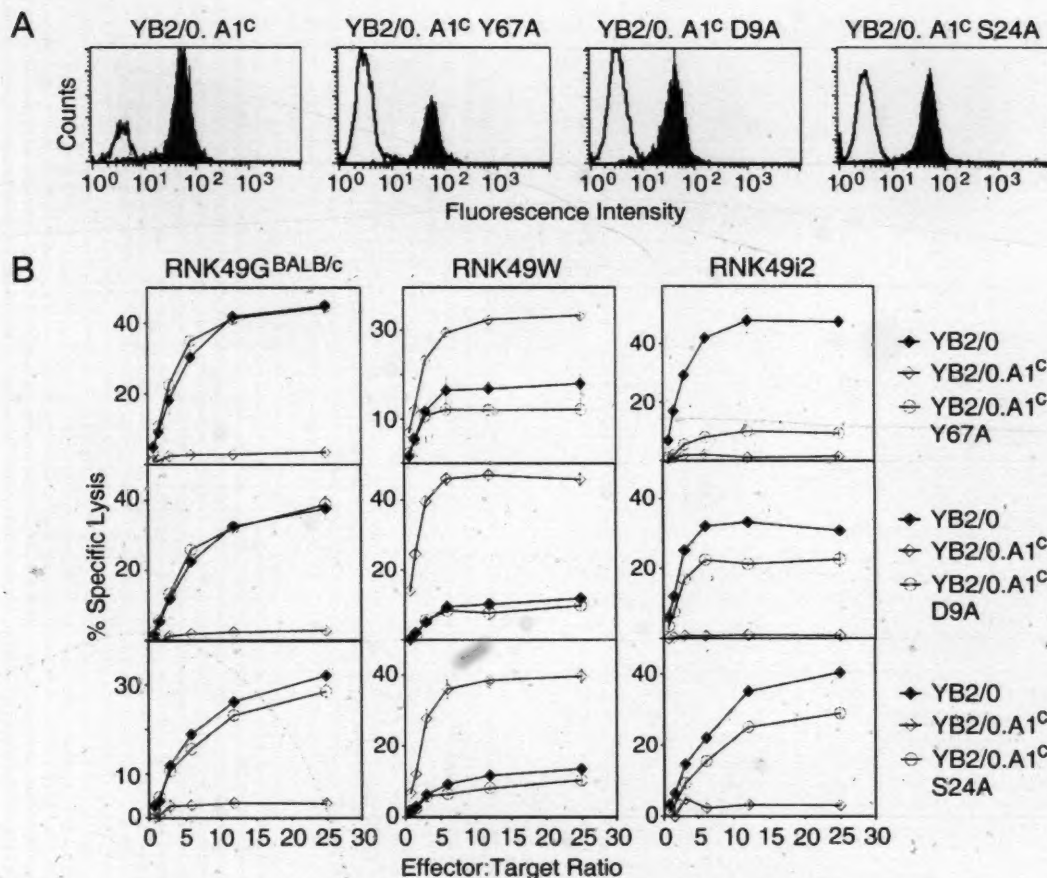


**Figure 3-3.** Xenogeneic and syngeneic Ly49 recognition of RT1-A1<sup>c</sup> is sensitive to alterations in the anchor residue binding B-pocket of the MHC I molecule. *A*, Wild-type RT1-A1<sup>c</sup> and its B-pocket mutants Y67A, D9A and S24A were each expressed to similar levels on YB2/0 cells. Transfected YB2/0 cells (*shaded histograms*) were assessed for RT1-A1<sup>c</sup> specific YR5/12 antibody staining compared to untransfected YB2/0 cells (*open histograms*). *B*, RNK-16 cells transfected with Ly49G<sup>BALB/c</sup>, Ly49W or Ly49i2 were compared for cytotoxicity against YB2/0 and YB2/0 transfected with the indicated RT1-A1<sup>c</sup> B-pocket mutants or wild-type RT1-A1<sup>c</sup>. Data represent the mean of triplicate wells  $\pm$  SD and are representative of three independent experiments.

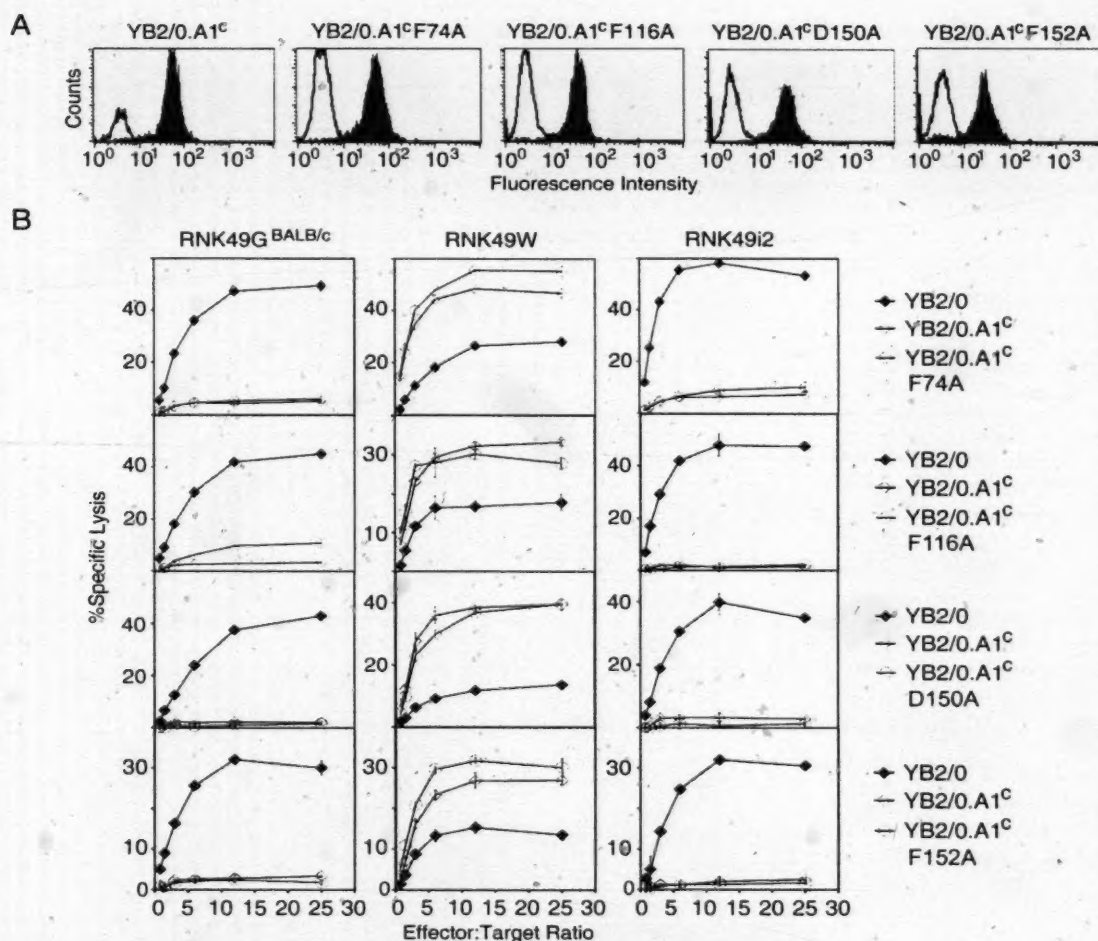


**Figure 3-4.** Xenogenic and syngeneic Ly49 recognition of RT1-A1<sup>c</sup> is not sensitive to alterations in the peptide-binding groove outside of the anchor binding B-pocket of the MHC I molecule. *A*, Wild-type RT1-A1<sup>c</sup> and its peptide-binding groove mutants F74A, F116A, D150A and F152A were each expressed to similar levels on YB2/0 cells. Transfected YB2/0 cells (*shaded histograms*) were assessed for RT1-A1<sup>c</sup> specific YR5/12 antibody staining compared to untransfected YB2/0 cells (*open histograms*). *B*, RNK-16 cells transfected with Ly49G<sup>BALB/c</sup>, Ly49W or Ly49i2 were compared for cytotoxicity against YB2/0 cells transfected with the indicated RT1-A1<sup>c</sup> peptide-binding groove mutants and wild-type RT1-A1<sup>c</sup>. Data represent the mean of triplicate wells  $\pm$  SD and are representative of three independent experiments.

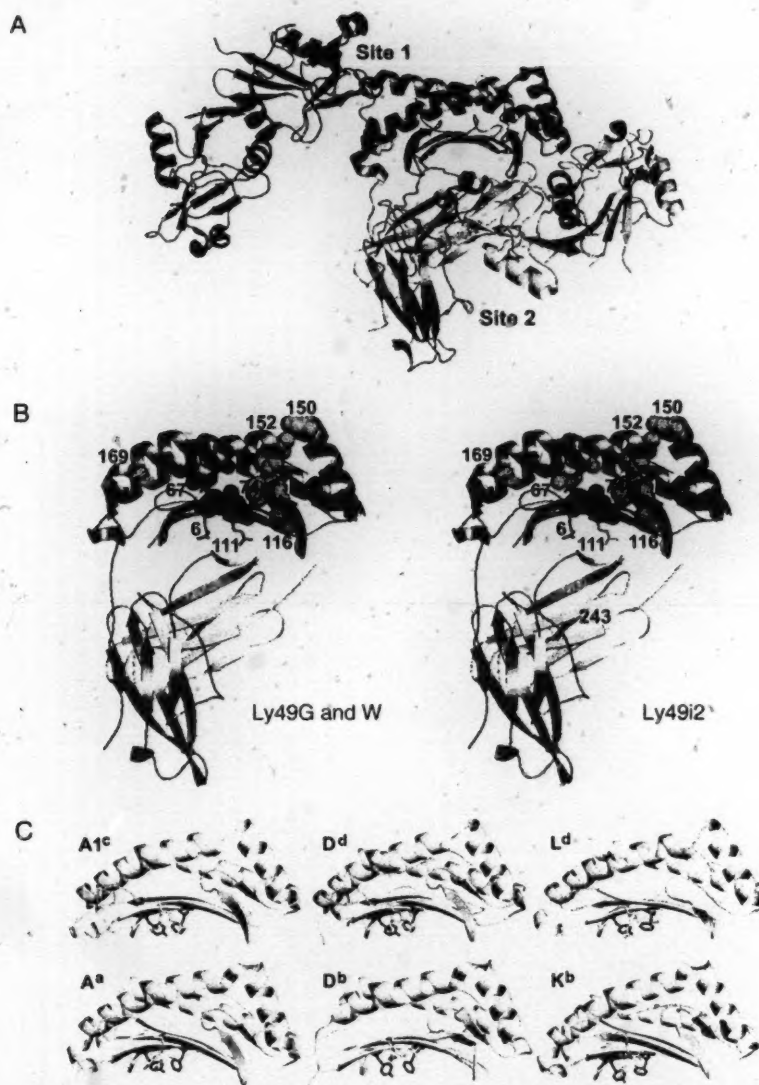


**Figure 3-5.** Alterations in the polymorphic and supertype defining B-pocket of RT1-A1<sup>c</sup> disrupts interaction with Ly49 receptors, possibly by altering the conformation of solvent exposed residues at site 2. *A*, Co-crystal of Ly49A (violet and teal) and H-2D<sup>d</sup> (blue) showing site 1 and site 2 interactions, respectively. *B*, Depiction of residues mutated in RT1-A1<sup>c</sup> and comparison of their effects on xenogeneic Ly49G and W recognition (*left*) versus syngeneic Ly49i2 recognition (*right*). Total disruption, red; no disruption, green; partial disruption, orange; double mutant disruption, purple. Residues within the peptide-binding groove are shown as spheres, solvent exposed residues as ball and stick. *C*, Comparison of the conformation of solvent exposed residues, shown in ball and stick, which articulate with the B-pocket of ligands (RT1-A1<sup>c</sup>, H-2D<sup>d</sup>, L<sup>d</sup>) versus non-ligands (RT1-A<sup>a</sup>, H-2D<sup>b</sup>, K<sup>b</sup>). Side chains are depicted in blue except for R6 and R111, which are depicted in yellow.



Table 3-1. P2 residues of peptides eluted from wild-type and mutant RT1-A1<sup>c</sup> molecules

| RT1-A1 <sup>c</sup> |                     | MHC I Molecule <sup>a</sup> |           |                     |           |                      |           |
|---------------------|---------------------|-----------------------------|-----------|---------------------|-----------|----------------------|-----------|
|                     |                     | A1 <sup>c</sup> Y67A        |           | A1 <sup>c</sup> D9A |           | A1 <sup>c</sup> S24A |           |
| P2 <sup>b</sup>     | %Yield <sup>c</sup> | P2                          | %Yield    | P2                  | %Yield    | P2                   | %Yield    |
| <b>P</b>            | <b>52</b>           | <b>P</b>                    | <b>45</b> | <b>P</b>            | <b>31</b> | <b>Q</b>             | <b>29</b> |
| L                   | 11                  | Q                           | 17        | V                   | 23.5      | <b>P</b>             | <b>26</b> |
| V                   | 9                   | L                           | 14        | R                   | 16.5      | E                    | 12        |
| G/Q                 | 8/8                 | I                           | 12        | T/Q                 | 11/11     | N                    | 11        |
| A/I                 | 5/5                 | V                           | 7         | K                   | 7         | V                    | 8         |
| F                   | 2                   | E/F                         | 2/2       |                     |           | I/T                  | 7/7       |
|                     |                     | Y                           | 1         |                     |           |                      |           |

<sup>a</sup> His tagged and purified from YB2/0 transfectants using Ni-NTA beads.

<sup>b</sup> Residues found at position 2 as determined by Edman degradation of peptides eluted from wild-type RT1-A1<sup>c</sup> or the designated MHC I mutants. Residues found in >25% of eluted peptides (in bold) were considered as dominant anchor residues.

<sup>c</sup> The P2 protein molar yield is expressed as the percentage of total molar yield from amino acids showing a pmol increase at P2.

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## CHAPTER IV

### DISTINCT INTERACTIONS WITH MULTIPLE SITE 2 SUBSITES BY ALLELE SPECIFIC RAT AND MOUSE LY49 DETERMINE BINDING AND CLASS I MHC SPECIFICITY<sup>1,2</sup>

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#### A. Introduction

Natural killer (NK) cells are large granular lymphocytes that play an important role in mediating innate resistance to transformed and virally infected cells, through both the secretion of soluble mediators and cytolytic activity (1). Interactions between NK cells and potential target cells occur through a myriad of activating and inhibitory receptors on the NK cell surface (2). In murine species, such as the rat and mouse, these include members of the Ly49 lectin-like receptor family (3-5).

Ly49 receptors belong to polygenic families comprised of numerous members both in the mouse and the rat (6, 7). Inbred rat and mouse strains each have a characteristic *Ly49* haplotype (8-10), and allelic variation between *Ly49* has been identified between mouse strains (11, 12). Within *Ly49* gene families both activating and inhibitory receptor types are found, with some activating/inhibitory hybrid receptors

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identified in the rat (7). Class I MHC (MHC I) is the demonstrated ligand for most well studied activating and inhibitory murine Ly49 (4, 13). Additionally, the viral MHC I homolog, m157 from mouse cytomegalovirus, is a ligand for the mouse activating Ly49H and inhibitory Ly49I receptors (14).

Recognition of MHC I by Ly49 is allele specific, with each Ly49 receptor able to recognize only one or a small repertoire of MHC I alleles. In inbred rodent strains, each NK cell expresses only a small subset of the available genes in its *Ly49* haplotype (15) but often at least one inhibitory Ly49 capable of recognizing an autologous MHC I allele product, which then transmits a dominant suppressive signal to the NK cell, thereby maintaining NK cell tolerance to self (16). Loss or lack of self-MHC I through viral infection, or transformation would then release the NK cell from this dominant suppression, precipitating NK effector functions toward the target cell (17).

The molecular interaction between mouse Ly49 and MHC I has been studied by mutagenesis (18-24) and examination of co-crystal structures of Ly49A/H-2D<sup>d</sup> and Ly49C/H-2K<sup>b</sup> (25, 26), and occurs primarily at a large interface known as "site 2" that encompasses MHC I residues below the peptide binding groove, within the  $\alpha 3$  domain, and on the  $\beta 2$ -microglobulin ( $\beta 2m$ ). What has remained poorly understood is the mechanism by which this site 2 interface, which is highly conserved both within and between rat and mouse MHC I alleles, can engender the observed allele specificity of Ly49 receptors.

Recently, we demonstrated that one of the polymorphic pockets within the peptide binding groove of MHC I, that serves to secure the peptide in the groove through specific "anchor residues" in the peptide, and that defines the supertype of the MHC I molecule, can determine allele specific recognition of MHC I (27). We hypothesized that this may occur through conformational alterations in solvent exposed residues at site 2 that directly articulate with the anchor binding and supertype defining pockets of MHC I, thereby defining which polymorphic Ly49 would then be able to interact with critical residues at this site (27). This reliance on supertype defined conformations for allele specificity would allow NK cells to survey for the presence of MHC I superotypes on the cell surface, about nine of which are found in species as diverse as rodent and primate through

convergent evolution (28, 29), and which appear critical for CTL recognition and function (30, 31).

To investigate whether allele specific Ly49 differentially interact with solvent exposed residues at site 2, possibly due to conformational alterations caused by anchor-binding and supertype-defining pockets and to investigate if rat Ly49 recognition of MHC I occurs in a similar manner to mouse, we examined both a rat and a mouse Ly49/MHC I allele specific receptor and ligand combination, creating mutations of solvent exposed residues at site 2 that articulate directly with the anchor binding pockets of MHC I. Because co-crystal structures of Ly49A with H-2D<sup>d</sup> (25) and Ly49C with H-2K<sup>b</sup> (26) predict that each of these Ly49/MHC I alleles interact with different residues in the  $\alpha 3$  domain of MHC I (Fig. 4-1A and B) we also mutated  $\alpha 3$  domain residues to determine whether conformational differences in MHC I might also affect how Ly49 receptors are oriented in relation to the  $\alpha 3$  domain, generating altered dependency on residues in this domain for recognition.

Our data leads us to present a model in which three heavy chain subsites, at the highly conserved site 2 of MHC I, function in combination to determine both rat and mouse Ly49 binding and allele specificity for MHC I. The F-subsite, which involves solvent exposed residues articulating with the more conserved anchor binding F-pocket, acts in both species as an anchor point and interacts with highly conserved residues in Ly49. The B-subsite, involving residues that articulate with the polymorphic, supertype defining and N-terminal peptide anchor binding pocket(s) on MHC I, directly interacts with the polymorphic L3 loop of Ly49 but may additionally contribute to allele specificity by altering placement of the Ly49 at site 2, likely owing to variable  $\beta 2m$  orientation. The altered positioning at site 2 then makes residues in the  $\alpha 3$  domain (C-subsite) of MHC I more or less available for interaction with polymorphic  $\beta 4$ - $\beta 5$  and  $\beta 2$ - $\beta 2'$  loops in Ly49. This model appears to apply in both the rat and mouse, and is consistent with convergent evolution in these two species to produce an innate recognition system capable of scrutinizing cells for the presence of MHC I supertypes, which are so crucial to CTL function and adaptive immunity.

## B. Materials and Methods

### *Hybridomas and monoclonal antibodies*

Hybridomas producing the antibodies 4D11 (rat IgG2a), anti-Ly49G<sup>BALB/c</sup> (32) and 34-5-8S (IgG2a), anti-H-2D<sup>d</sup> were obtained from American Type Culture Collection (Manassas, VA). Antibodies were prepared from ammonium sulfate precipitates as described (33). Purified STOK2 (rat IgG2a), anti-Ly49i2 antibody (34, 35) was purchased from BD Biosciences Pharmingen (San Diego, CA). RT1-A1<sup>c</sup> specific YR5/12 (rat IgG2b) hybridoma supernatant (36), was purchased from Serotec (Oxford, UK). The R-Phycoerythrin-conjugated secondary antibodies, AffinPure F(ab')<sub>2</sub> Fragment Donkey Anti-Rat IgG, and AffinPure F(ab')<sub>2</sub> Fragment Donkey Anti-Mouse IgG, were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

### *Cell lines*

YB2/0, a non-secreting rat myeloma, was obtained from American Type Culture Collection and was maintained in DMEM supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, 1mM sodium pyruvate and 0.1mM non-essential amino acids. RNK-16, a spontaneous F344 rat strain NK cell leukaemia cell line (37), was maintained in RPMI supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and 5 x 10<sup>-5</sup>M 2-mercaptoethanol. All transfected RNK-16 and YB2/0 cells were maintained under G418 selection. Transfected cells were grown in the absence of G418 for at least 48 hours prior to cytotoxicity assays.

### *Cloning and transfection of Ly49i2*

Total RNA was isolated from 1x10<sup>7</sup> PVG rat LAK cells using an RNeasy Protect mini-kit (Qiagen, Valencia, USA). cDNA was produced using Powerscript reverse transcriptase (Clontech, Palo Alto, USA) with an oligo(dT) primer. Ly49i2 was amplified with Advantage-HF 2 polymerase mix using primers designed from the sequence NM\_152848 published by Naper et al. (35), digested with XhoI/XbaI and ligated into BSRαEN (Dr. A. Shaw, Washington University, St. Louis, USA). RNK-16 cells were transfected as previously described (38).



### *Mutagenesis of MHC I*

RT1-A1<sup>c</sup>, previously cloned in this laboratory from PVG rat spleen (39) and H-2D<sup>d</sup> from S49.1 T lymphoma cells (33), were mutated using a QuikChange Mutagenesis Kit (Stratagene, La Jolla, USA). Mutagenic primers were designed to mutate to alanine, potential solvent exposed interaction residues directly below or near the B-pocket (residues 6, 8, 10, 23, 27 and 98), below the F-pocket (residues 115 and 122) and in the  $\alpha$ 3 domain of the MHC-CI heavy chain (residues 223, 232, 243, 262) as defined by Ly49A/H-2D<sup>d</sup> and Ly49C/H-2K<sup>b</sup> co-crystals (25, 26). Double mutants in the B-subsite (R6A F8A), F-subsite (Q115A D122A), C-subsite (E232AY262A, D223AK243A, E232AK243A, Y262AK243A) and between subsites R6AD122A, R6AK243A, D122AK243A) were generated through an additional round of mutagenesis on RT1-A1<sup>c</sup> constructs already containing the primary mutation. All mutations were verified by DNA sequencing. Mutagenesis was performed on RT1-A1<sup>c</sup> directly within the H-2D<sup>k</sup> leader-enhanced green fluorescence protein (EGFP) fusion vector that was previously generated in this laboratory (39). H-2D<sup>d</sup> was subcloned into the EGFP fusion vector with its native leader sequence intact and mutagenesis performed directly on this construct. YB2/0 cells were stably transfected with each construct as previously described (33).

### *Flow cytometric analysis*

Successful transfection of MHC I/EGFP fusion constructs was determined by EGFP fluorescence intensity of transfected YB2/0 cells relative to untransfected YB2/0 cells in 96 well black walled plates using a FLA-5100 Imaging System (Fujifilm, Allendale, NJ). Proper folding and surface expression levels were determined using specific primary antibody to RT1-A1<sup>c</sup> (YR5/12) and H-2D<sup>d</sup> (34-5-8S) plus relevant PE-conjugated secondary antibody. Expression of the Ly-49i2 and Ly49G<sup>BALB/c</sup> receptors by RNK-16 cells was monitored compared to untransfected RNK-16 cells using primary antibodies STOK2 and 4D11, respectively, followed by FITC-conjugated relevant secondary antibody. Surface expression was assessed using a FACScan flow cytometer (BD Biosciences, Mountain View, CA). Target cells were assayed on the same day as cytotoxicity assays for matched surface expression of mutant and control MHC I.



### Cytotoxicity Assays

Target cells were labeled with 100-150  $\mu$ Ci of  $\text{Na}^{51}\text{CrO}_4$  ( $^{51}\text{Cr}$ ) (Mandel, Guelph, Canada) at 37°C for 1 hour. After washing targets three times with RPMI they were plated at  $1 \times 10^4$   $^{51}\text{Cr}$ -labelled cells per well and mixed with RNK-16 or transfected RNK-16 cells in V-bottom microtitre plates at indicated E:T ratios in triplicate. After a 4 hour incubation at 37°C, plates were centrifuged for 5 minutes at 1500 rpm and 25  $\mu$ l of supernatant was collected and counted in a MicroBeta TriLux liquid scintillation counter (PerkinElmer, Wellesley, USA). Percent specific lysis was determined as (experimental release – spontaneous release)/(maximal release – spontaneous release) x 100%.

### C. Results

*Ly49G recognition of H-2D<sup>d</sup> is disrupted by mutagenesis of single residues articulating with anchor binding pockets at subsite-B, whereas Ly49i2 recognition of RT1-AI<sup>c</sup> is unaffected*

Solvent exposed residues at site 2 of the MHC I molecule are highly conserved between MHC I alleles in both mouse and rat, and even between the two species, making it difficult to understand how Ly49 receptors exhibit allele specificity for MHC I when interacting with this site. Recently, we demonstrated that the polymorphic, P2 peptide anchor residue binding and supertype defining B-pocket is important in determining Ly49 allele specificity for MHC I (27). We proposed that this may occur through polymorphism-induced conformational alterations of side chains on solvent exposed amino acids below and articulating with the B-pocket, thereby altering their availability for interaction with Ly49. In support of this, Ly49 recognition has been shown to be reliant on R6 of MHC I, a residue that lies below and articulates directly with the B-pocket, in a number of allele combinations (19, 27). These observations suggested that allele specific MHC I recognition by Ly49 may involve variable usage of solvent exposed MHC I residues below and articulating with the polymorphic B- (or N-terminal peptide anchor binding) pocket(s), at what we have designated as the B-subsite (Fig. 4-1A).

When Dam et al. (26) solved the co-crystal of H-2K<sup>b</sup> and Ly49C they showed that the Ly49C/H-2K<sup>b</sup> interface, while having some Ly49/MHC I residue interactions in

common with the Ly49A/H-2D<sup>d</sup> co-crystal interface (25), involves a unique set of residues on the MHC I molecule and unique Ly49/MHC I residue interactions to mediate recognition. This appears to be due to both the different orientation of Ly49C on H-2K<sup>b</sup> and polymorphisms in Ly49C. The unique orientation of Ly49C on H-2K<sup>b</sup> seemed to be due to a different mode of dimerization for Ly49C compared to Ly49A. In the Ly49C co-crystal, the Ly49C dimer was more "open" allowing one Ly49C receptor to symmetrically bind two H-2K<sup>b</sup> molecules simultaneously whereas in the Ly49A co-crystal, Ly49A dimerization was more "closed" resulting in an asymmetrical binding orientation on a single H-2D<sup>d</sup> molecule. Ly49A has since been crystallized in a similar "open" dimerization state, capable of binding two H-2D<sup>d</sup> molecules simultaneously (40), making variable Ly49 dimerization states unlikely mediators of allele specificity. Regardless, variable placement of the Ly49 receptor on the MHC I molecule and allele specific residue usage at the B-subsite and two other subsites that we have identified on the MHC I heavy chain (Fig. 4-1A and B), may contribute to allele specificity at the highly conserved site 2 of MHC I. These two additional subsites include subsite-C, involving interaction residues on the  $\alpha$ 3 domain of the MHC I heavy chain and subsite-F, involving solvent exposed residues beneath and articulating with the anchor binding F-pocket, which is typically more conserved in amino acid composition than the B-pocket (Fig. 4-1A and B).

We set out to investigate the usage of residues at each subsite by two different Ly49/MHC I allele combinations, for comparison with data previously collected on the Ly49A/H-2D<sup>d</sup> and Ly49C/H-2K<sup>b</sup> allele combinations, to determine if variable MHC I residue usage at one or more subsites could be mediating allele specificity of Ly49 for MHC I. One combination, H-2D<sup>d</sup> and the BALB/c allele of Ly49G2, allowed us to compare residue usage at each subsite by a Ly49 that is related to, but polymorphic from Ly49A, and exhibits similar specificity for the H-2D<sup>d</sup> allele. The other allele combination included a classical rat MHC I molecule, RT1-A1<sup>c</sup>, and the rat Ly49i2 inhibitory receptor. This combination allowed us to investigate whether rat, like mouse, showed similar reliance on site 2 for recognition and, for the first time, to map the residues mediating Ly49 recognition of MHC I in this species. RNK-16 cells transfected to express the rat Ly49i2 or mouse Ly49G inhibitory receptors (Fig. 4-1C) were used as

effector cells toward YB2/0 target cells and YB2/0 transfected to express their respective MHC I ligands, RT1-A1<sup>c</sup> and H-2D<sup>d</sup> (Fig. 4-1D) in 4-h cytotoxicity assays (Fig. 4-1E). RNK-16 effector cells expressing the inhibitory Ly49i2 or Ly49G receptors exhibit cytotoxicity toward untransfected YB2/0 cells but are inhibited from killing transfected YB2/0 targets through recognition of their respective RT1-A1<sup>c</sup> and H-2D<sup>d</sup> MHC I ligands (Fig. 4-1E), as previously reported (12, 35).

Alterations of the polymorphic and supertype defining B-pocket in MHC I disrupt Ly49 recognition (27), possibly by altering the conformation of solvent exposed residues that specifically articulate with the N-terminal peptide anchor binding pocket(s) of MHC I. We therefore mutated to alanine, individual solvent exposed residues that articulate directly with the anchor-binding B-pocket of RT1-A1<sup>c</sup> (R6, F8) (Fig. 4-2A) and the anchor binding B- and C-pockets of H-2D<sup>d</sup> (R6, F8, M98) (Fig. 4-2B) along with residues that may also contribute to Ly49 interaction below the peptide-binding groove but that do not directly articulate with anchor binding pockets. For RT1-A1<sup>c</sup>, these residues included I10, I23, Y27 and M98 (Fig. 4-2A) and for H-2D<sup>d</sup>, residues T10, M23 and Y27 (Fig. 4-2B). Since both the B- and C-pockets act as the supertype defining and anchor binding pockets of H-2D<sup>d</sup> we have grouped/designated solvent exposed residues below and articulating with these pockets of H-2D<sup>d</sup> as the B-subsite. Each mutant was expressed on YB2/0 cells to similar levels compared to wild type RT1-A1<sup>c</sup> (Fig. 4-3A) and H-2D<sup>d</sup> (Fig. 4-3B) MHC I molecules. RT1-A1<sup>c</sup>, and each of its mutant forms, exhibited similar abilities to inhibit cytotoxicity of YB2/0 through Ly49i2 recognition of the transfected MHC I molecules (Fig. 4-3C). In contrast, Ly49G recognition of H-2D<sup>d</sup> was greatly reduced by the R6A mutant, as previously seen for Ly49A recognition of this molecule (19), and was completely disrupted by the F8A and M98A mutations of H-2D<sup>d</sup> (Fig. 4-3D). All of these residues lie directly beneath and normally articulate with the anchor-binding B- and C-pockets of H-2D<sup>d</sup> (Fig. 4-2B). The Y27A mutant of H-2D<sup>d</sup> exhibited a partial loss in ability to inhibit cytotoxicity in repeated assays (Fig. 4-3D and *data not shown*), possibly reflecting its position below but on the periphery of the B-pocket (Fig. 4-2B) and therefore a less crucial interaction residue at the B-subsite. Mutagenesis of residues T10 and M23, which are positioned in the middle of the peptide-binding platform and away from the anchor-binding B- and C-pockets, showed no effect on Ly49G recognition of H-



2D<sup>d</sup> as would be expected if only residues below and articulating with the anchor-binding pockets were required for recognition at this subsite (Fig. 4-3D). In summary, Ly49i2 recognition of RT1-A1<sup>c</sup> and Ly49G recognition of H-2D<sup>d</sup> differ in their reliance on solvent exposed residues at subsite-B with Ly49i2 recognition being resistant to single mutations at this subsite, despite a demonstrated reliance on the B-pocket for recognition (27), and Ly49G recognition being specifically sensitive to alterations in residues directly below and articulating with the anchor-binding B- and C-pockets of H-2D<sup>d</sup> (Fig. 4-2A and B).

*Ly49i2 and Ly49G both exhibit sensitivity to alterations in solvent exposed residues below and articulating with the F-pockets of RT1-A1<sup>c</sup> and H-2D<sup>d</sup> at subsite-F*

Since we have shown that allele specific Ly49 recognition of MHC I appears to specifically rely on residues both within (27) and articulating with the anchor-binding and supertype-defining B-pocket, the latter at least for H-2D<sup>d</sup> recognition by Ly-49G (Fig. 4-3D) and since Ly49A/H-2D<sup>d</sup> and Ly49C/K<sup>b</sup> recognition is apparently highly dependent on the solvent exposed residues Q115 and D122 (19, 25, 26) located below the other peptide-anchoring F pocket at what we designated the F-subsite, we decided to investigate whether Q155 and D122 might also play a significant role in rat Ly49i2 and mouse Ly49G recognition of MHC I. RT1-A1<sup>c</sup> was mutated to alanine at Q115 and D122 (Fig. 4-2A) and each mutant was expressed to a similar level as wild-type RT1-A1<sup>c</sup> on YB2/0 target cells (Fig. 4-4A). H-2D<sup>d</sup> was also mutated at residues Q115 and D122 (Fig. 4-2B) but only the Q115A mutant could be expressed to comparable levels compared to wild-type H-2D<sup>d</sup> (Fig. 4-4A and *data not shown*). The inability to express the D122A mutant was likely due to the previously demonstrated reliance of H-2D<sup>d</sup> on D122 for association with the peptide loading complex (41). The Q115A mutant of RT1-A1<sup>c</sup> showed no loss of recognition by Ly49i2 compared to wild-type RT1-A1<sup>c</sup> (Fig. 4-4B), whereas the single D122A mutant showed partial disruption of recognition by Ly49i2 in repeated assays using different clones, all with equivalent expression levels to wild-type RT1-A1<sup>c</sup> (Fig. 4-4B and *data not shown*). In contrast, Ly49G recognition of H-2D<sup>d</sup> was nearly completely disrupted through mutagenesis of the Q115 residue at subsite-F and exhibited similar levels of cytolysis to untransfected YB2/0 cells in the same assay (Fig.



4-4C). In contrast to the B-subsite, it is evident that both Ly49i2 and Ly49G, similar to Ly49A and C, rely on solvent exposed residues that articulate with the more conserved anchor binding F-pocket, at the F-subsite, for recognition of MHC I, with Ly49G recognition of H-2D<sup>d</sup> again exhibiting greater sensitivity to a single residue alteration at this subsite compared to Ly49i2 recognition of RT1-A1<sup>c</sup> (Fig. 4-2A and B).

*Specific single amino acid changes at subsite C in the MHC I  $\alpha$ 3 domain, can completely abolish Ly49G recognition of H-2D<sup>d</sup>, whereas Ly49i2 recognition of RT1-A1<sup>c</sup> is relatively unaffected*

The co-crystal structures of Ly49C/H-2K<sup>b</sup> (26) and Ly49A/H-2D<sup>d</sup> (25) predict that each of these Ly49/MHC I allele combinations interact with a different set of residues in the highly conserved  $\alpha$ 3 domain of MHC I. Therefore, we designated this as another potential subsite, subsite-C, for the discrimination of MHC I alleles by Ly49. We mutated H-2D<sup>d</sup> and RT1-A1<sup>c</sup> at amino acid positions corresponding to those previously identified in co-crystal structures and/or through mutagenesis as involved in either Ly49A recognition of H-2D<sup>d</sup> (E232, K243) or Ly49C recognition of H-2K<sup>b</sup> (Y262, E223, K243) (25, 26) to assess if Ly49i2 and Ly49G recognition of their respective MHC I ligands also depended on the same or different sets of residues at subsite-C. Each mutant in RT1-A1<sup>c</sup> (D223A, E232A, K243A and E262A) (Fig. 4-2A) was expressed in YB2/0 cells at similar cell surface levels compared to wild type RT1-A1<sup>c</sup>, as were the H-2D<sup>d</sup> C-subsite mutants (E223A, E232A, K234A, E262A) (Fig. 4-2B) compared to wild type H-2D<sup>d</sup> (Fig. 4-5A). Cytolysis by RNK-16 cells expressing Ly49i2 remained inhibited through recognition of each RT1-A1<sup>c</sup> single mutant at the C-subsite, compared to wild type RT1-A1<sup>c</sup> expressed on YB2/0 cells. One possible exception being YB2/0 cells expressing the E232A mutant, which consistently showed a modest increase in cytolysis compared to wild type RT1-A1<sup>c</sup> (Fig. 4-5B). In contrast, Ly49G recognition of H-2D<sup>d</sup> showed greater sensitivity to single mutants at this subsite. RNK-16 cells expressing Ly49G had continued, although slightly reduced, recognition and cytolytic inhibition of YB2/0 cells expressing the E223A and E262A H-2D<sup>d</sup> C-subsite mutants, compared to wild type H-2D<sup>d</sup> in the same assay (Fig. 4-5C). However, Ly49G recognition of H-2D<sup>d</sup> was totally disrupted by mutagenesis of either E232 or K243 residues to alanine (Fig. 4-

5C). Therefore, H-2D<sup>d</sup> recognition by Ly49G again shows different and greater sensitivity to single mutants at this subsite compared to Ly49i2 recognition of RT1-A1<sup>c</sup>, with Ly49i2 recognition of RT1-A1<sup>c</sup> being slightly disrupted by mutagenesis at E232 and Ly49G recognition of H-2D<sup>d</sup> being slightly impaired through E223A and E262A mutations and completely disrupted by mutagenesis at E232 and K243 (Fig. 4-2A and B).

*Ly49i2 recognition of RT1-A1<sup>c</sup> is sensitive to combined alanine mutations at two different subsites*

Since Ly49i2 recognition of RT1-A1<sup>c</sup> showed little or no disruption by mutagenesis of single residues at each of the B-, F- and C-subsites there was the possibility that rat Ly49/MHC I interactions do not rely on the same interaction site, or subsites as mouse Ly49/MHC I. Based on our previous results showing that Ly49i2 recognition of RT1-A1<sup>c</sup> relies on residues within the anchor binding B-pocket (27) and the partial loss of recognition seen with the D122A (Fig. 4-4B) and E232A mutants (Fig. 4-5B) it seemed unlikely that Ly49i2 recognition relied on entirely different subsites on MHC I compared to mouse Ly49. Therefore, we made combined alanine mutations in RT1-A1<sup>c</sup> at two different subsites. We chose the residues R6, D122 and K243 that Matsumoto et al. previously mutated in H-2D<sup>d</sup> demonstrating that Ly49A recognition occurs at site 2 (19) as these residues are found at subsite-B, -F and -C, respectively (Fig. 4-2A). The three double mutants generated, R6A D122A, D122A K243A and R6A K243A were expressed on YB2/0 cells to similar levels as wild type RT1-A1<sup>c</sup> (Fig. 4-6A). The R6A D122A mutant, which represents mutations at both the B- and F-subsites, completely disrupted recognition by Ly49i2 (Fig. 4-6B). This indicated that the partial loss of recognition seen with the single D122A mutant at subsite-F (Fig. 4-4B) could be augmented through mutagenesis of the B-subsite that previously appeared to have no effect. Similarly, pairing mutations at the F-subsite and C-subsite as with the D122A K243A mutant also produced an augmentation of the partial loss of recognition seen with the single D122A mutant (Fig. 4-4B) and resulted in near complete loss of recognition (Fig. 4-6B) indicating that, despite the ineffectiveness of single mutations at the C-subsite, that this subsite indeed plays a role in the recognition of RT1-A1<sup>c</sup> by Ly49i2. The most compelling evidence for the importance of B- and C-subsites in mediating

recognition by Ly49i2 came with the B/C-subsite mutant R6A K243A, which was also able to disrupt recognition of RT1-A1<sup>c</sup> (Fig. 4-6B) despite the inability of single mutations at either subsite to disrupt recognition individually (Figs. 4-3C and 5B).

Therefore, rat Ly49i2 recognition, like mouse Ly49 recognition of MHC I, also occurs at site 2 and relies on the B-, F- and C-subsites in combination, for recognition of RT1-A1<sup>c</sup> (Fig. 4-6C).

*Ly49i2 recognition of RT1-A1<sup>c</sup> is sensitive to double alanine mutations at each individual subsite*

Having established that Ly49i2 recognition of RT1-A1<sup>c</sup>, like mouse Ly49 recognition of MHC I, also relies on site 2 and the same B-, F- and C-subsites, we wanted to assess more fully the usage of residues at each individual subsite for comparison to the information already gathered for mouse alleles. Since we were able to detect partial loss of Ly49i2 recognition using single mutations at subsites-F and -C of RT1-A1<sup>c</sup> (Figs. 4-2A, 4B and 5B) and since concurrent mutations at two different subsites were able to disrupt recognition (Fig. 4-6), it appeared that Ly49i2, while relying on similar subsites as mouse Ly49, might interact with greater overall affinity for its MHC I ligand.

In order to assess more fully the usage of residues at each subsite we produced double alanine mutations at each subsite of RT1-A1<sup>c</sup> that could possibly then overcome this evidently greater affinity of Ly49i2 for its ligand, providing additional information on the usage of each individual subsite on RT1-A1<sup>c</sup> (Fig. 4-7A). At the B-subsite, we mutated residues R6 and F8 (Fig. 4-7A) since both these residues strongly affected Ly49G recognition of H-2D<sup>d</sup> (Fig. 4-3D) and because both articulate closely with the anchor binding B-pocket of the RT1-A1<sup>c</sup> molecule. Additionally, the R6 mutation was able to disrupt recognition in combination with mutants at other subsites (Fig. 4-6B). At the F-subsite we chose D122 as it has a partial effect as a single mutant in RT1-A1<sup>c</sup> (Fig. 4-4B) and combined it with Q115 (Fig. 4-7A), which had an effect on Ly49G recognition of H-2D<sup>d</sup> (Fig. 4-4C). Lastly, at the C-subsite we chose mutant combinations that would either have an effect in a Ly49A/H-2D<sup>d</sup> (25) or Ly49G/H-2D<sup>d</sup> type interaction, relying on residues E232A and K243A in the  $\alpha$ 3 domain for recognition (Fig. 4-7B) or in a



Ly49C/H-2K<sup>b</sup> type interaction (26) where residues D223A and E262A, and not E232, act in addition to K243 to mediate recognition (Fig. 4-7C).

Each double subsite mutant was expressed on YB2/0 cells to similar cell surface expression levels as wild type RT1-A1<sup>c</sup> (Fig. 4-8A) and used as targets in 4-h cytotoxicity assays with RNK-16 effector cells expressing the Ly49i2 inhibitory receptor (Fig. 4-8B). The B-subsite continued to show a reduced importance for recognition of RT1-A1<sup>c</sup> by Ly49i2 as YB2/0 cells expressing the double B-subsite mutant, R6A F8A, showed only a partial loss of recognition compared to YB2/0 cells expressing wild type RT1-A1<sup>c</sup> in the same assay (Fig. 4-8B). In contrast, at the F-subsite, the partial disruption caused by the single D122A RT1-A1<sup>c</sup> mutant seen in Figure 4-4B was augmented by the addition of the Q115A mutation, that alone had no effect (Fig. 4-4B), resulting in complete loss of recognition by Ly49i2 expressing RNK-16 cells (Fig. 4-8B). Lastly, double RT1-A1<sup>c</sup> mutants at the C-subsite also demonstrated a role for this subsite for recognition by Ly49i2 (Fig. 4-8B). In examination of the Ly49C/H-2K<sup>b</sup> interaction type mutants, D223A E262A and D223A K243A, it appeared that Ly49i2 did not use this type of interaction for recognition of RT1-A1<sup>c</sup>, as YB2/0 cells expressing both of these C-subsite double mutants were recognized to a similar degree as wild type RT1-A1<sup>c</sup> expressing YB2/0 cells (Fig. 4-8B). Expression of the Ly49A,G/H-2D<sup>d</sup> interaction type mutant, E232A K243A on YB2/0 resulted in a loss of recognition by Ly49i2 expressing RNK-16 cells, suggesting that the Ly49i2 receptor may follow a similar pattern as Ly49A and G for recognition of MHC I at the C-subsite (Fig. 4-8B), and is substantiated by the partial loss of recognition of the single E232A mutant seen in Figure 4-5B. Further investigation with the Ly49C/H-2K<sup>b</sup> interaction type mutant, E262A K243A revealed that Ly49i2 also relies on this residue combination for recognition at the C-subsite, as YB2/0 cells expressing this mutant were lysed at similar levels to untransfected YB2/0 cells (Fig. 4-8B). Therefore, Ly49i2 recognition of RT1-A1<sup>c</sup> appears to have a Ly49A/G and Ly49C hybrid-type dependence on residues at subsite-C (Fig. 4-7).

The use of double mutants at each subsite reveals that like the mouse Ly49/MHC I allele combinations, rat Ly49i2 also interacts with MHC I at site 2 and utilizes a combination of residues at the B-, F- and C-subsites in recognition of the RT1-A1<sup>c</sup> MHC I allele (Fig. 4-7A). Together, both mouse and rat Ly49 recognition of MHC I appears to



rely on a combination of three subsites on the MHC I heavy chain for recognition. Comparisons of the mutagenesis results for the Ly49/MHC I combinations investigated here, along with mutagenesis data and co-crystal structure predictions previously collected for additional Ly49/MHC I allele combinations, suggest that unique placement of the Ly49, possibly imposed by anchor pocket mediated conformational differences between MHC I, and the resulting variable residue usage at MHC I subsites, could be the mechanism mediating allele specificity between polymorphic Ly49 and the highly conserved recognition site 2 on MHC I.

#### **D. Discussion**

We demonstrate here that rat Ly49 recognition of a rat classical MHC I molecule occurs at site 2 in a manner resembling mouse Ly49/MHC I recognition. Additionally, we contribute extensive mutagenesis analyses of both a rat and a new mouse Ly49/MHC I allele combination, Ly49i2/RT1-A1<sup>c</sup> and Ly49G/H-2D<sup>d</sup>, respectively, to the mutagenesis and crystallographic data available for Ly49C recognition of H-2K<sup>b</sup> (26, 42-44) and Ly49A recognition of H-2D<sup>d</sup> (18-20, 22, 25, 45) allowing us to compare the usage of solvent exposed residues at three subsites of the conserved site 2 interaction site on MHC I in four different Ly49/MHC I allele combinations.

Rat Ly49i2 and mouse Ly49G recognition of MHC-I ligands showed differential degrees of dependence on subsite-B, which includes residues that extend below and articulate with the N-terminal peptide anchor binding and supertype defining pocket(s) of H-2D<sup>d</sup> and RT1-A1<sup>c</sup> (Fig. 4-2). Ly49G was highly dependent on this site for recognition, being disrupted by mutations of individual amino acids that articulate solely with the anchor binding B- and C- pockets of the H-2D<sup>d</sup> molecule. This included R6, which is also important in Ly49A recognition of H-2D<sup>d</sup> (19, 25) and the previously unexamined F8 and M98 that also articulate with the supertype-defining B- and C-pockets but did not include solvent exposed residues that articulate with non-anchor binding regions within the peptide binding groove (Fig. 4-2B). In contrast, RT1-A1<sup>c</sup> recognition by the rat Ly49i2 receptor was unaffected by single mutations at this subsite (Fig. 4-2A). Despite this, Ly49i2 recognition of RT1-A1<sup>c</sup> shared at least some reliance on residues below and articulating with the anchor binding B-pocket of MHC I, as demonstrated through double

mutants at the B-subsite or a single mutation at this subsite in combination with a mutation at an additional subsite (Fig. 4-6C and 7A). Our results in conjunction with analysis of the Ly49A/D<sup>d</sup> (25) and Ly49C/K<sup>b</sup> (26) crystal structures (Fig. 4-9) suggest why Ly49G (like Ly49A) and Ly49i2 (like Ly49C) may behave somewhat differently in their recognition of this subsite. Both Ly49A and Ly49G share a highly similar sequence in the L3 loop region with 18 of 21 residues sharing identity. Of the three polymorphisms between Ly49A and Ly49G in L3 (residues R/G214, R/T223 and G/L231, respectively) only residue 223 appears potentially important since the NTT223 motif in Ly49G, as opposed to the NTR223 motif in Ly49A, introduces a carbohydrate attachment site on Ly49G. The absence of R223 and addition of carbohydrate on Ly49G could alter how Ly49G interacts with H-2D<sup>d</sup> compared to Ly49A in two ways. First, it would preclude the interaction seen between Ly49A and H-2D<sup>d</sup> through R223 on Ly49A and E104 on H-2D<sup>d</sup> (25) which is a minor interaction between Ly49A and H-2D<sup>d</sup> as demonstrated through mutagenesis (18, 20). Moreover, the addition of carbohydrate at the MHC I/Ly49 interface due to the presence of the NTT motif in Ly49G that is not found in Ly49A, has potential steric effects and has been shown to affect the affinity of Ly49G for H-2D<sup>d</sup> (46). What is in common between Ly49A and G in L3 is D229, which in combination with the overall identity in L3 between the two receptors, likely mediates the reliance of Ly49G/D<sup>d</sup> interaction on residue R6 in the MHC I molecule at subsite-B (22, 25) (Fig. 4-9A). In contrast, Ly49i2 differs from both Ly49A and G by 16 and 17 of 21 residues in L3. Ly49C is also significantly different from the Ly49A-like subfamily of Ly49 receptors (6) in L3, and is predicted from its co-crystal structure with H-2K<sup>b</sup> not to make a significant number of contacts using this loop (Fig. 4-9B), Ly49i2 may too rely only slightly, or differently, on the L3 loop for recognition requiring less direct contact with solvent exposed residues at subsite-B.

While the reliance of Ly49G and i2 on residue R6 at the B-subsite of MHC I, is likely due to direct interaction with the polymorphic L3 of the Ly49, inspection of crystal structures suggest that the reliance on additional residues articulating with the anchor binding pocket(s) would appear to be due to their interaction with  $\beta$ 2m, which has previously been demonstrated to be an important interface for recognition by Ly49 (22-26). Residue F8 of RT1-A1<sup>c</sup> and both F8 and M98 of H-2D<sup>d</sup>, while articulating directly

with the N-terminal anchor binding and supertype defining pocket(s) of MHC I, also interact with the evolutionarily conserved F56 and W60 residues of  $\beta 2m$  (Fig. 4-10). This motif forms the major interface between  $\beta 2m$  and MHC I heavy chains and is responsible for the variable orientation of  $\beta 2m$  seen between MHC I alleles (47-51). Intriguingly, upon examination of the crystal structures of H-2D<sup>d</sup> and RT1-A1<sup>c</sup>, the main F56 and W60 interaction residues in  $\beta 2m$  lie directly below the anchor binding pockets of MHC I (Fig. 4-10), possibly explaining how the polymorphic anchor binding pockets of MHC I could affect Ly49 recognition through conformational changes; not only by making solvent exposed residues on the MHC I heavy chain more or less available for direct interaction with Ly49, as may be the case for R6, but also through positioning of the  $\beta 2m$ . In support of this,  $\beta 2m$  is known to rotate its core around its F56, W60 interaction loop, through interaction with polymorphic residues, particularly in anchor binding pockets of MHC I (26, 47, 52). In some cases,  $\beta 2m$  orientation depends on the loaded peptide's sequence (53, 54), and it has been suggested that this may explain the overt reliance of Ly49C/I on peptide sequence for recognition of MHC I (26, 55-57). This may also explain our previous observation that rat Ly49i2 and mouse Ly49G and W are sensitive to subtle changes both in the MHC I anchor binding pockets, and possibly in the usage of specific peptide anchor residues in peptides bound to RT1-A1<sup>c</sup> (27).

Beta-2 microglobulin also makes contact with residues that articulate with the anchor binding F-pocket of MHC I, including residue Q115 mutated in this study (26) (Fig. 4-10) and may also contribute somewhat to the loss of Ly49 recognition upon mutagenesis of this residue at subsite-F. Additionally, subsite-F residues are known to contribute directly to the interaction with mouse Ly49, particularly D122, which is responsible for the vast majority of atomic contacts, 23 in total, between H-2D<sup>d</sup> and Ly49A (22) (Fig. 4-9A). Both available co-crystals (25, 26) indicate that the interaction of Ly49A and C with H-2D<sup>d</sup> and K<sup>b</sup>, respectively, is reliant on a conserved 236, 238, 239 triad in mouse Ly49 (Fig. 4-9), and Dam et al. suggested that these residues provide the majority of free energy to the interaction (26, 58); a concept that is also supported through Ly49A mutagenesis studies (22). This triad is also conserved in many rat Ly49 and similarly, rat Ly49i2 recognition of RT1-A1<sup>c</sup> was most strongly reliant on the F-



subsite, as the D122A mutant was the only single mutant able to produce a significant loss in recognition (Fig. 4-2A). In Ly49, S236 in particular is highly conserved between both mouse and rat Ly49 and is likely responsible, along with residue T238, for the reliance of Ly49i2 on residue D122. Although the D122A mutant of H-2D<sup>d</sup> remained untested with Ly49G due to expression difficulties, it is highly likely that this mutant would also have disrupted recognition, as it does Ly49A recognition of H-2D<sup>d</sup> (19), due to the highly conserved nature of the triad between these mouse Ly49. Ly49C and A are predicted to be reliant on Q115 of their MHC I ligands solely through interaction with R239 (25, 26) (Fig. 4-9), which is also highly conserved in rat and mouse Ly49, including Ly49G that we demonstrated required Q115 for recognition, but interestingly is polymorphic in Ly49i2. This R239G polymorphism in Ly49i2 may explain both the inability to disrupt recognition by the single Q115A mutant of RT1-A1<sup>c</sup> as well as the only partial disruption of RT1-A1<sup>c</sup> recognition by mutation of D122, a residue that also forms bonds with R239 in both available co-crystals (Fig. 4-9). The very close association of Q115 in RT1-A1<sup>c</sup> with W60 in  $\beta$ 2m may be the additional means by which the double mutant of Q115A D122A was able to completely disrupt Ly49i2 recognition at this subsite (Fig. 4-10).

The third heavy chain subsite we investigated was subsite-C in the  $\alpha$ 3 domain of MHC I. We again made mutants in this subsite on H-2D<sup>d</sup> and RT1-A1<sup>c</sup> based on the predictions of Ly49C and A co-crystals (25, 26) and tested their effects on Ly49G and i2 recognition. Ly49i2 again showed resistance to single mutations at this subsite with the exception of a slight decrease in recognition of the E232A mutant of RT1-A1<sup>c</sup> (Fig. 4-2A). Despite this, Ly49i2, like mouse Ly49, appeared reliant on interactions at this subsite, being disrupted by double mutations between subsite-C and another subsite (Fig. 4-6C) or within the  $\alpha$ 3 domain (Fig. 4-7A). In particular, Ly49i2 required residues 232, 243 and 262 for recognition of MHC I (Fig. 4-7A) exhibiting a hybrid-type reliance on residues at the C-subsite compared to residues 223, 243, 262 predicted for Ly49C (26) and 232, 243 for Ly49A (Fig. 4-9) (19, 25). The region(s) of Ly49 that primarily interact with subsite-C, the  $\beta$ 2- $\beta$ 2' loop (Fig. 4-9B residues 203, 204) and the  $\beta$ 4- $\beta$ 5 loop (Fig. 4-9A residues 244, 246, 247), are polymorphic, particularly the  $\beta$ 4- $\beta$ 5 loop. Interestingly, Ly49G and Ly49i2 share the identical DCGK  $\beta$ 4- $\beta$ 5 loop sequence previously shown to



be important for recognition at this site (59) and share a demonstrated reliance on residues 232 and 243 of MHC I for recognition (Fig. 4-2B and 7A), likely through K247 and D244 of this sequence, respectively. Why Ly49i2 shows an additional reliance on E262 in RT1-A1<sup>c</sup> is difficult to explain without a co-crystal structure but could be mediated by the variable positioning of the  $\alpha 3$  domain seen between MHC I alleles (50, 54, 60), by the variable orientation of  $\beta 2m$  discussed previously, or variable interaction with  $\beta 2m$  through polymorphic Ly49i2 residues at the  $\beta 2m$  interface, such that the polymorphic  $\beta 2$ - $\beta 2'$  loop that mediates this interaction between Ly49C/K<sup>b</sup> (Fig. 4-9B) becomes positioned to mediate an interaction between lysine residues in the  $\beta 2$ - $\beta 2'$  loop of Ly49i2 and E262 of RT1-A1<sup>c</sup>.

The possibility that Ly49 become variably oriented on the  $\alpha 3$  domain of MHC I to mediate interactions at subsite-C is further supported by the differential sensitivities of Ly49G and Ly49A to H-2D<sup>d</sup> mutants and comparison of the  $\beta 2$ - $\beta 2'$  and  $\beta 4$ - $\beta 5$  loop motifs of each Ly49. We demonstrate Ly49G sensitivity to alanine mutations of K243 and E232 and to a slight degree on E223 and E262 (Fig. 4-2B) while Ly49A exhibits primary reliance on K243 and despite its predicted importance in the Ly49/H-2D<sup>d</sup> co-crystal, is not disrupted by E232 mutation (19) (Fig. 4-9A). These sensitivities are potentially explainable by the substitution of the DcGK  $\beta 4$ - $\beta 5$  motif of Ly49G for the NcDQ motif of Ly49A and a very slight rotation of Ly49G in relation to Ly49A on H-2D<sup>d</sup> such that K243 and E232 of H-2D<sup>d</sup> form interactions with D244 and K247 of Ly49G, respectively. Additionally, the Ly49A/H-2D<sup>d</sup> co-crystal demonstrates that the  $\beta 2$ - $\beta 2'$  loop, which is identical between Ly49A and G, is not available for Ly49A interaction with residues E262 or D223 on H-2D<sup>d</sup> (Fig. 4-9A), whereas the slight rotation of Ly49G required to place D244 in contact with K243 of H-2D<sup>d</sup> in a manner similar to the predicted D244 usage by Ly49C (Fig. 4-9B), could bring the Ly49G  $\beta 2$ - $\beta 2'$  loop into closer range for interaction at E262 and D223, similar to the interaction seen in the Ly49C/H-2K<sup>b</sup> co-crystal (Fig. 4-9B). Since orientation of the H-2D<sup>d</sup>  $\alpha 3$  domain and  $\beta 2m$  is likely to be identical in both scenarios, the slight rotation suggested in Ly49G recognition could occur through the addition of carbohydrate at T223 and/or polymorphisms that exist between Ly49G and A at the  $\beta 2m$  interface. Ly49C also

interacts with a unique set of residues at the C-subsite (26, 44) and this appears to occur through a differential orientation of the Ly49 to  $\beta 2m$  and the  $\alpha 3$  domain (26, 48) (Fig. 4-9B).

It has been suggested through domain substitution (4, 18, 61, 62) and mutagenesis (18-21, 45) studies that the  $\alpha 2\alpha 3$  domain and peptide binding groove are particularly important for allele specific Ly49 recognition of MHC I through undefined conformational differences in the MHC I heavy chain. Additionally, it has been suggested that peptide specificity of Ly49C for H-2K<sup>b</sup> (56) may occur through subtle effects on floor residues in H-2K<sup>b</sup> that are transmitted to Ly49C through  $\beta 2m$  (26). To date no one has proposed a clearly defined mechanism by which this could occur. We propose that the conformational change in MHC I could be mediated primarily through the highly polymorphic anchor pocket(s) in MHC I that bind the N-terminal peptide anchor residue(s) (27). This would not only alter the direct availability of solvent exposed residues below the pocket(s) for interaction at subsite-B (27) but would variably orientate  $\beta 2m$  (26, 47, 52), altering the placement of polymorphic Ly49 loops on the  $\alpha 3$  domain of MHC I, and possibly on  $\beta 2m$  itself. Such a mechanism would put particular emphasis on the polymorphic L3,  $\beta 2$ - $\beta 2'$  and  $\beta 4$ - $\beta 5$  regions of Ly49 (Fig. 4-9) in mediating allele specificity, whereas the conserved 236, 238, 239 triad of Ly49 (Fig. 4-9) would provide a stable interaction with the more conserved F-pocket and solvent exposed residues of the F-subsite. This is consistent with our findings here showing that mutagenesis of F8 and M98, residues that articulate specifically with both the N-terminal anchor binding pockets of H-2D<sup>d</sup> and  $\beta 2m$  and could have a large impact on  $\beta 2m$  orientation (Fig. 4-10), affect Ly49G recognition of H-2D<sup>d</sup>. Additionally, our findings along with previous findings indicate that each of the four Ly49 examined use a different "set" of residues at the C-subsite of MHC I for recognition, indicating differential placement of Ly49 on the  $\alpha 3$  domain of MHC I. This is also in agreement with the suggestion of Dam et al. (26) that the conserved triad of Ly49 mediates primary recognition of all MHC I alleles through contacts with Q155 and/or D122, contributing most of the binding free energy, while variable and polymorphic regions of Ly49 might determine allele specificity (26, 58). Such a mechanism provides an explanation not only for the overt peptide selectivity seen

for Ly49C/I recognition of MHC I (55-57), since peptide has been demonstrated to change the orientation of  $\beta 2m$  on the ligands for these receptors (53, 54), but also for the loss of MHC I recognition by Ly49 upon subtle changes in the anchoring B-pocket or anchor residue dominance of peptides bound to RT1-A1<sup>c</sup> molecules (27). It may also explain the xenoreactivity we saw between Ly49G and RT1-A1<sup>c</sup>, but not other rat MHC I (39), as RT1-A1<sup>c</sup> is unique among the molecules we tested in that it shares a similar supertype and  $\beta 2m$  orientation with H-2D<sup>d</sup> (Fig. 4-2 and 10). Furthermore, Ly49G and Ly49i2 have similar  $\beta 4$ - $\beta 5$  loop motifs that upon similar, possibly supertype and  $\beta 2m$  mediated, placement on the  $\alpha 3$  domain of RT1-A1<sup>c</sup> could mediate similar subsite-C interactions.

While we are able to demonstrate with a high degree of certainty that rat Ly49, like mouse, interacts at the highly conserved site 2 interface of MHC I and relies on a combination of the B- F- and C-subsites for recognition, one has to take note that Ly49i2 recognition was much more resilient to single mutations in MHC I compared to Ly49G, A or C (18-20, 22, 42-45). Ly49i2 shows significant polymorphisms, at all potential interfaces with RT1-A1<sup>c</sup> compared to Ly49A/G recognition of H-2D<sup>d</sup> or Ly49C recognition of H-2K<sup>b</sup> that could account for the inability of single mutations designed from available mouse crystal structures to disrupt recognition in a rat system. This may have resulted in the mutagenesis of residues only secondary in importance for recognition at each subsite, similar to E232 usage depicted in the Ly49A/H-2D<sup>d</sup> co-crystal that has no effect on recognition in mutagenesis studies (19, 25). Despite this, we were particularly able to show a similar strong reliance of rat recognition on the F-subsite, which appears so pivotal to mouse Ly49/MHC I interactions. Like the highly conserved S236, T238, R239 triad in mouse, rat Ly49 either have this same triad or slightly altered triads such as the S236, T238, G239 triad that is found in Ly49i2 (7), which could slightly modify the usage of key residues at this subsite. Interestingly, the presence of two different triads for interaction with the F-subsite on rat MHC I may reflect the presence of two different TAP alleles in the rat capable of loading two different types of P9 anchor in the, therefore, slightly more polymorphic F-pocket of rat MHC I (63). This could possibly affect MHC I conformation and how Ly49 can interact with solvent exposed residues at the F-subsite, much like the conformational changes suggested for the more polymorphic



N-terminal peptide anchor binding pocket(s) at the B-subsite. This would be in contrast to mouse, which has a single TAP allele, restricted peptide P9 anchor residue repertoire and, therefore, likely a conserved F-subsite conformation (64). Ly49i2 and rat MHC I have other characteristics that may have affected overall Ly49i2 affinity for MHC I or reliance on specific "primary" residues capable of disrupting recognition as single heavy chain mutants. These include a much greater number of potential glycosylation sites on Ly49i2 compared to mouse Ly49, absence of carbohydrate on residue 176 of rat MHC I (55, 65), polymorphism in the  $\beta 0$  domain, important for Ly49 dimerization and allele specificity (22, 43), and Ly49 polymorphism at the interface with  $\beta 2m$  (22-26). Despite these differences, we show that rat and mouse Ly49 share a similar interaction site and reliance on these 3 subsites, particularly the primacy of the F-subsite in mediating recognition for all rat and mouse Ly49/MHC I combinations examined. With no clear orthologs between the rat and mouse Ly49 and MHC I molecules examined here, and an evolutionary gap of 20-40 million years, this mode of recognition clearly appears to have occurred through convergent evolution. It has been suggested that the conservation of key residues between rat and mouse  $\beta 2m$  has occurred through selective pressure to maintain  $\beta 2m$  residues important for Ly49 and CD8 interactions (24, 48). What would drive the convergent evolution of rat and mouse Ly49/MHC I recognition systems based on heavy chain conformations and  $\beta 2m$  orientations, possibly determined by polymorphic and supertype defining anchor pockets of MHC I, may be a similar requirement in both species to destroy cells that are unable to present a recognizable MHC I supertype to cytolytic T cells.

In summary, we demonstrate that mouse Ly49G and rat Ly49i2 rely on differential usage of residues at different subsites within the site 2 interface of MHC I molecules and in comparison with the co-crystals of Ly49A/H-2D<sup>d</sup> and Ly49C/H-2K<sup>b</sup>, demonstrate that variable residue usage at site 2 may be more than a by-product of differential modes of Ly49 dimerization between the Ly49A-like and C-like subfamilies (6). This becomes particularly probable upon the recent isolation of Ly49A in the "open" dimerization state, once thought only to be relevant for Ly49C-like receptors (40). Instead, the demonstrated differential interaction with residues at site 2 of the MHC I heavy chain by Ly49, likely along with variable Ly49 placement on  $\beta 2m$  and the  $\alpha 3$



domain, imposed by conformations dictated by anchor-binding and supertype-defining pocket(s), may be the mechanism by which allele specific Ly49 survey MHC I alleles, possibly for the presence of viable superotypes on the cell surface for recognition by TCR. This is a plausible evolutionary pressure where the convergent evolution of MHC I superotypes (28), fundamental to adaptive effector functions and crucial to a species' survival, may require simultaneous and rapid convergent evolution of the MHC I dependent innate effector functions that support them.

#### **E. Acknowledgments**

We thank Dong-Er Gong for excellent technical assistance.

#### **F. Author Contribution to Data**

Kerry Lavender generated the majority of data for this study. Heidi Chau assisted in the mutagenesis of RT1-A1<sup>c</sup> and H-2D<sup>d</sup> and transfection of YB2/0 cells. Heidi Chau also produced killing assay data used in Figures 4-3D and 4-5B and for the RT1-A1<sup>c</sup> D122A mutant in Figure 4-4B.

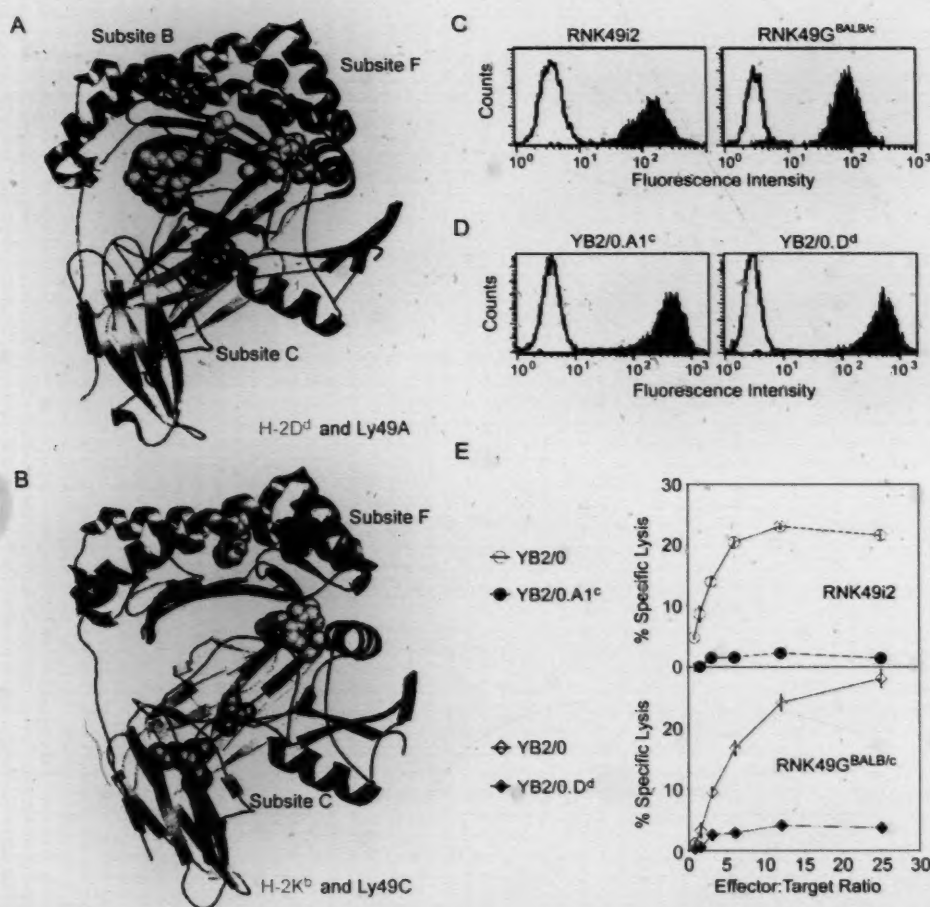


**Figure 4-1.** Designation of site 2 subsites and MHC I recognition by Ly49i2 and Ly49G. *A*, Co-crystal of Ly49A-2 (navy) with H-2D<sup>d</sup> (blue) and  $\beta$ 2m (grey) showing Ly49 residues interacting at subsites-B (yellow spheres), -C (green spheres) and -F (cyan spheres). *B*, Co-crystal of Ly49C-2 (navy) with H-2K<sup>b</sup> (blue) and  $\beta$ 2m (grey) showing Ly49 residues interacting primarily at subsites-C (green spheres) and -F (cyan spheres). Each subsite is demarcated by a grey ellipse and includes for subsites-B and -F, both anchor pocket and solvent exposed residues. N- and C-terminal peptide anchor residues are labelled and shown as pink and magenta spheres, respectively. *C*, RNK-16 cells transfected with Ly49i2 or Ly49G (shaded histograms), were stained with Ly49 specific Abs plus FITC-conjugated secondary Ab and compared to untransfected RNK-16 cells (open histograms). *D*, Surface expression of RT1-A1<sup>c</sup> and H-2D<sup>d</sup> on YB2/O cells was assessed using MHC I allele specific Abs plus secondary PE-conjugated Ab (shaded histograms) and compared to untransfected YB2/O cells (open histograms). *E*, YB2/O cells and YB2/O cells transfected with RT1-A1<sup>c</sup> or H-2D<sup>d</sup> were used as targets for RNK-16 cells expressing the inhibitory Ly49i2 or Ly49G receptor, respectively, in 4-h cytotoxicity assays. Data represent the mean of triplicate wells  $\pm$ SD and are representative of three independent experiments.

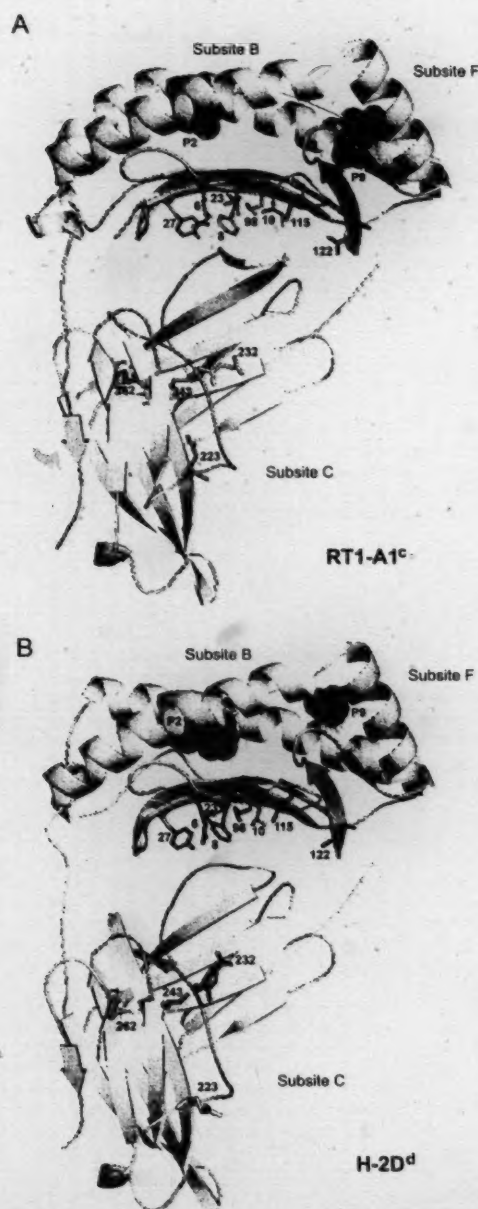


**Figure 4-2.** Mutagenesis of single residues at three MHC I subsites differentially effect Ly49i2 and Ly49G recognition of their ligands. Locations and effect of single alanine mutations at each subsite on Ly49i2 recognition of RT1-A1<sup>c</sup> (A) or Ly49G recognition of H-2D<sup>d</sup> (B). Subsites are demarcated by grey ellipses, complete disruption is indicated by red, partial disruption by shades of orange (darker shade indicates more disruption), and no disruption by green. The untested D122 residue in H-2D<sup>d</sup> is violet.



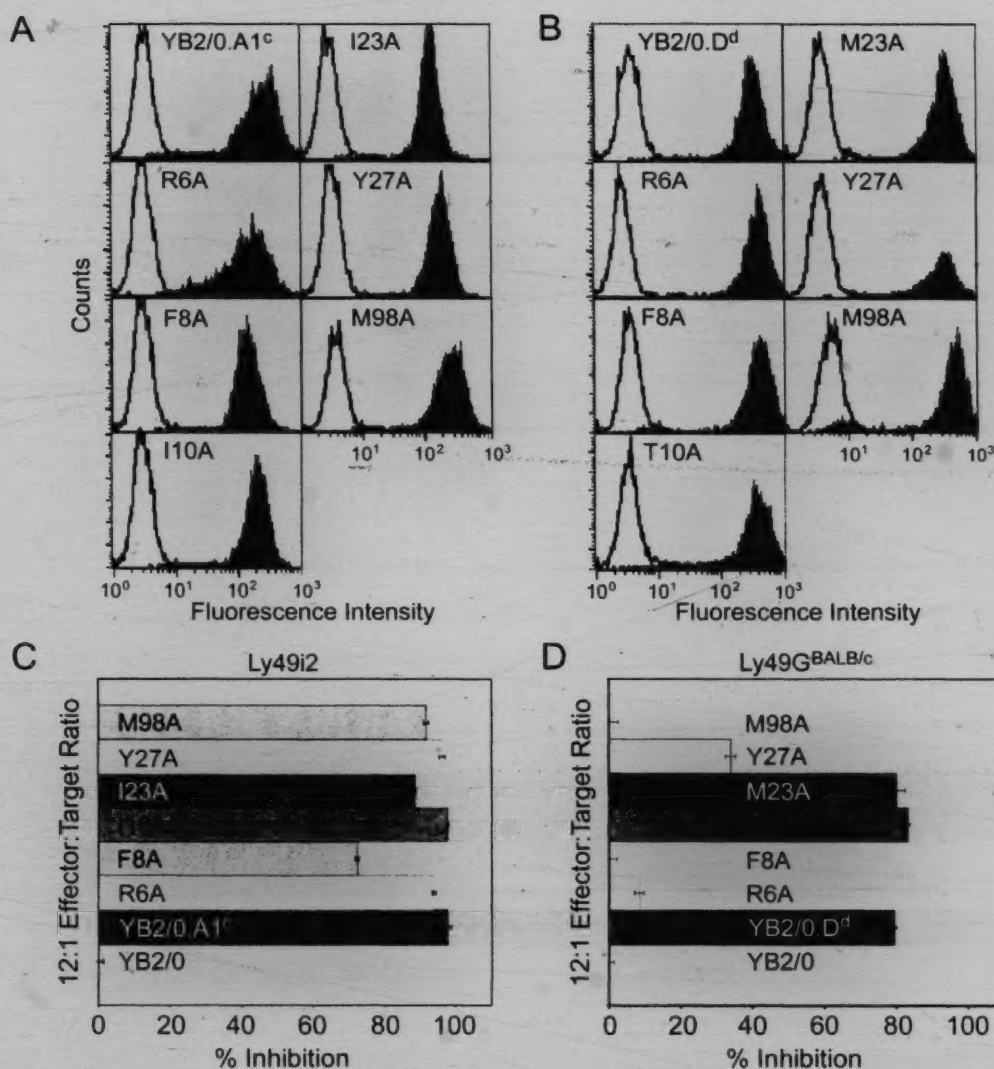


**Figure 4-3.** Ly49i2 recognition of RT1-A1<sup>c</sup> is unaffected by single B-subsite mutants whereas Ly49G recognition of H-2D<sup>d</sup> is disrupted by single mutants beneath the anchor binding B- and C-pockets at the B-subsite. *A*, RT1-A1<sup>c</sup> and H-2D<sup>d</sup> (*B*) or their respective single B-subsite mutants were expressed to similar levels on YB2/0 cells (*shaded histograms*) compared to untransfected YB2/0 cells (*open histograms*). YB2/0 cells expressing RT1-A1<sup>c</sup>, H-2D<sup>d</sup> (*D*) or their single B-subsite mutants were compared for their ability to inhibit cytolysis through recognition by RNK-16 cells expressing Ly49i2 (*C*) and Ly49G (*D*), respectively. Percent inhibition was calculated compared to untransfected YB2/0 in the same 4-h cytotoxicity assay. Percent specific lysis of YB2/0 by Ly49i2 and Ly49G RNK transfectants ranged between 20 and 40%. Data represent the mean of triplicate wells  $\pm$ SD and are representative of three independent experiments.

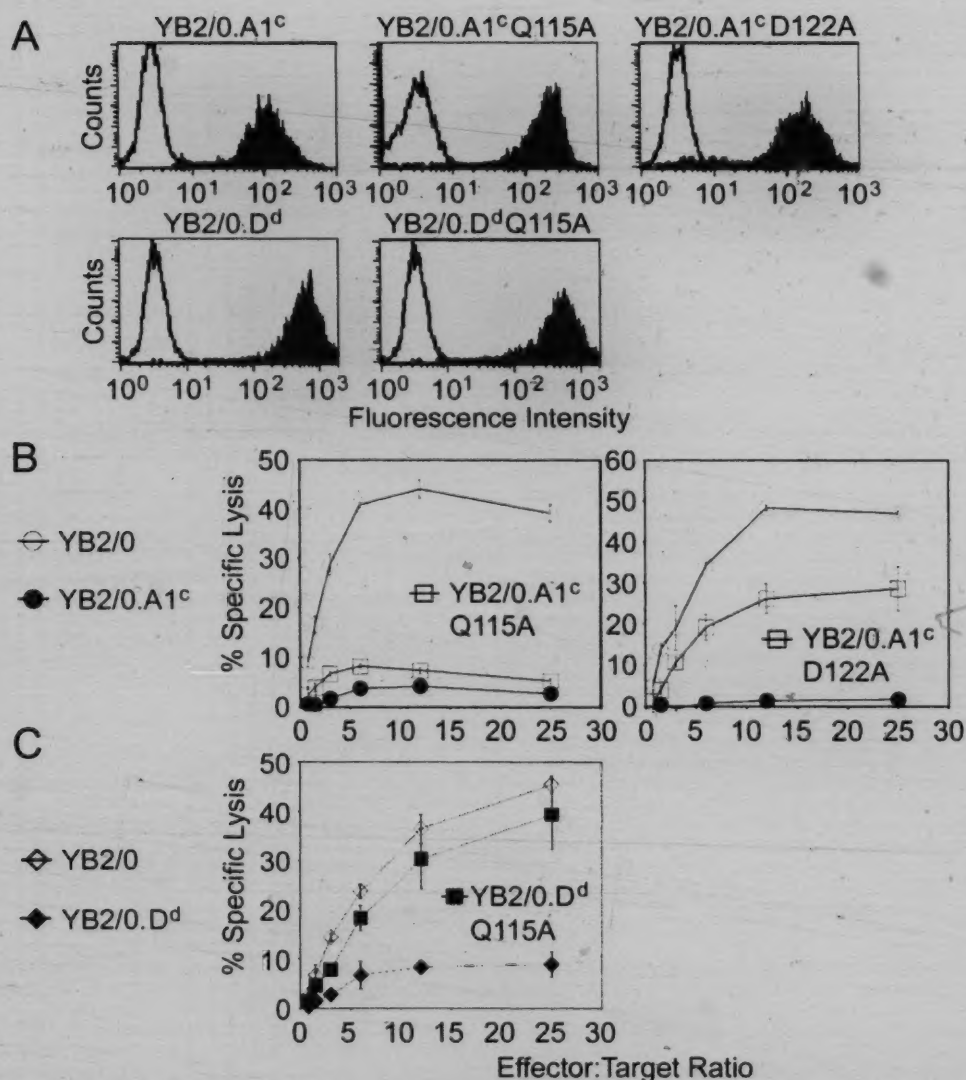


**Figure 4-4.** Single residue mutagenesis at the F-subsite completely disrupts Ly49G recognition of H-2D<sup>d</sup> and partially disrupts Ly49i2 recognition of RT1-A1<sup>c</sup> when at residue D122. *A*, YB2/0 cells expressing RT1-A1<sup>c</sup> and H-2D<sup>d</sup> were matched for expression with YB2/0 cells expressing their respective F-subsite mutants (*shaded histograms*) compared to untransfected YB2/0 cells (*open histograms*). F-subsite mutants of RT1-A1<sup>c</sup> (*B*) or H-2D<sup>d</sup> (*C*) were assayed for recognition by Ly49i2 and Ly49G, respectively, compared to untransfected and wild-type MHC I transfected YB2/0 cells in 4-h cytotoxicity assays. Data represent the mean of triplicate wells  $\pm$ SD and are representative of three independent experiments.

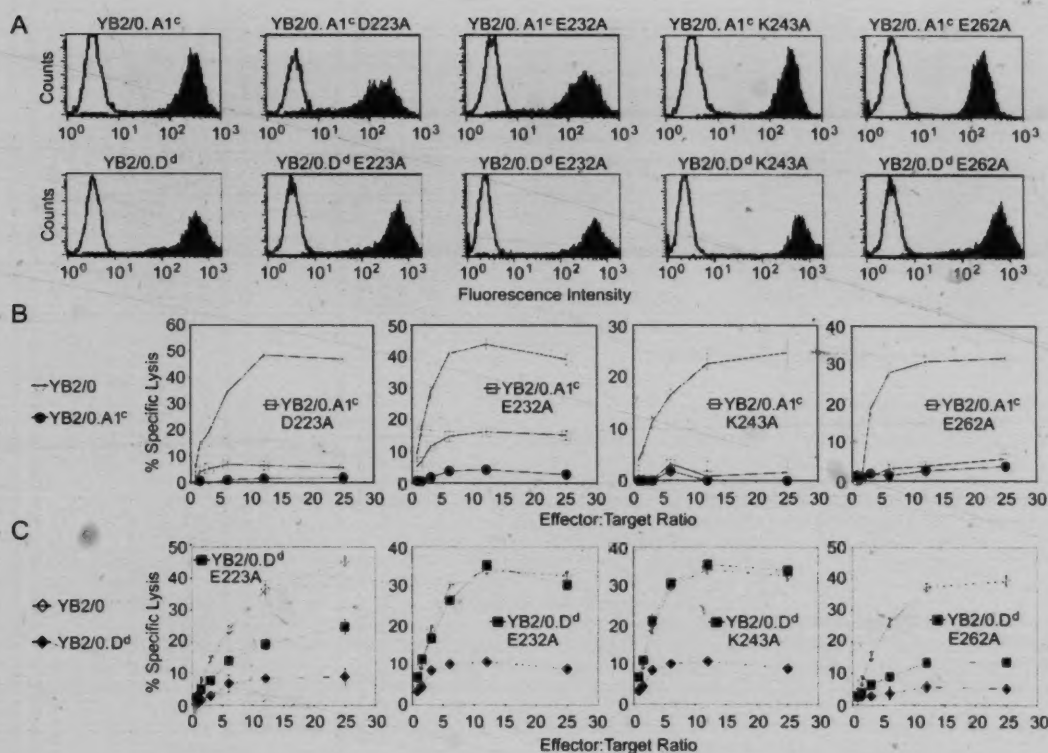


**Figure 4-5.** RT1-A1<sup>c</sup> recognition by Ly49i2 is relatively unaffected by single C-subsite mutations, while Ly49G recognition of H-2D<sup>d</sup> is sensitive to specific C-subsite point mutations. *A*, YB2/O cells expressing RT1-A1<sup>c</sup> and H-2D<sup>d</sup> C-subsite mutants were matched for expression with YB2/O cells expressing their respective wild-type molecule (*shaded histograms*) compared to untransfected YB2/O (*open histograms*). *B*, Ly49i2 recognition of RT1-A1<sup>c</sup> single C-subsite mutants and *C*, Ly49G recognition of H-2D<sup>d</sup> single C-subsite mutants in 4-h cytotoxicity assays. Results with respective wild-type MHC I and untransfected YB2/O controls are also indicated. Data represent the mean of triplicate wells  $\pm$ SD and are representative of three independent experiments.

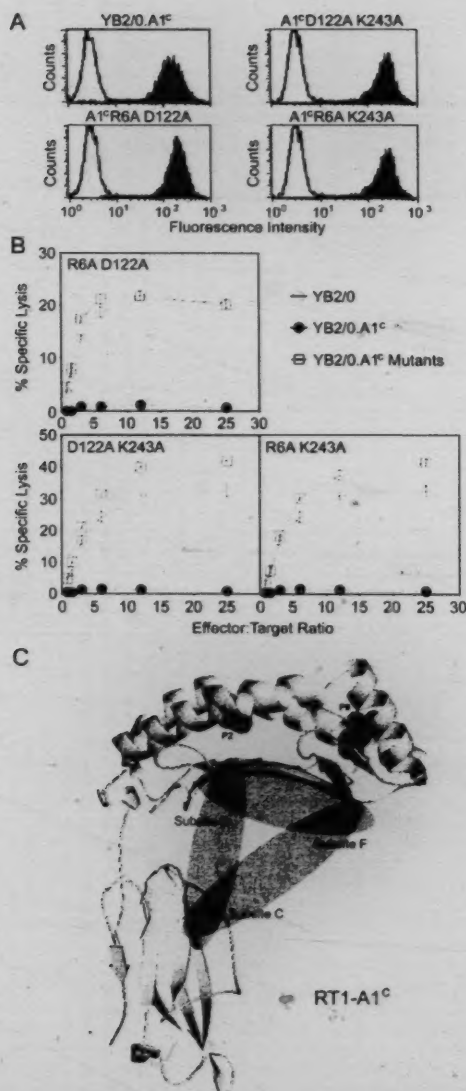


**Figure 4-6.** Recognition of RT1-A1<sup>c</sup> by Ly49i2 is completely disrupted by combined mutations at any two subsites. *A*, Double subsite mutants of RT1-A1<sup>c</sup> were expressed to similar levels as wild-type RT1-A1<sup>c</sup> on YB2/0 cells (*shaded histograms*) compared to untransfected YB2/0 cells (*open histograms*). *B*, YB2/0 cells expressing double subsite mutants of RT1-A1<sup>c</sup>, double B/F-subsite mutant, R6AD122A; double F/C-subsite mutant, D122AK243A and double B/C-subsite mutant, R6AK243A, were compared to untransfected or wild-type RT1-A1<sup>c</sup> expressing YB2/0 cells for recognition by Ly49i2 in 4-h cytotoxicity assays. Data represent the mean of triplicate wells  $\pm$ SD and are representative of three independent experiments. *C*, Location and effect of double alanine mutations at two separate subsites on Ly49i2 recognition of RT1-A1<sup>c</sup>. Complete disruption by a residue pair is depicted by shading of the residue pair in red ellipses. Anchor residues in the bound peptide are depicted as violet spheres.



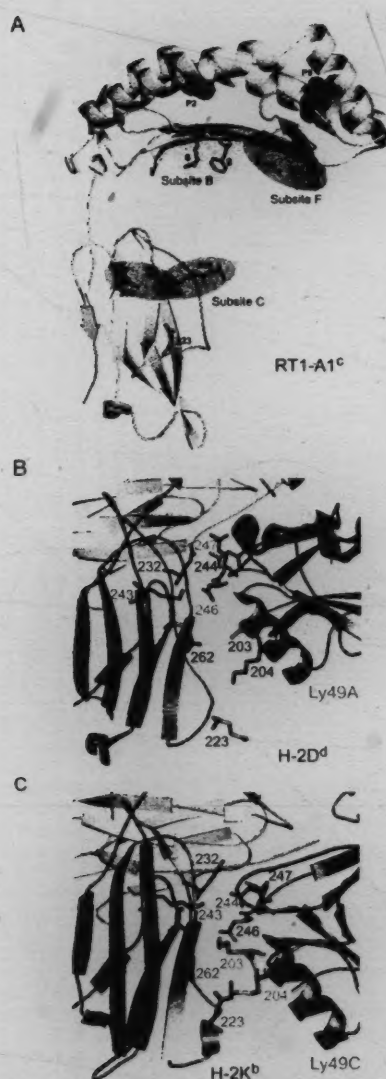


**Figure 4-7.** Ly49i2 recognition of RT1-A1<sup>c</sup> relies on each individual subsite at site 2, relying on a Ly49A/C hybrid-type residue usage at the C-subsite. *A*, Location and effect of double alanine mutations within a single subsite on Ly49i2 recognition of RT1-A1<sup>c</sup>. Complete disruption by a residue pair is depicted by shading of the residue pair in red ellipses, partial disruption by a residue pair by shading in orange. Anchor residues in the bound peptide are depicted as violet spheres. Close-up of subsite-C depicting the partially overlapping but distinct residues used in Ly49A recognition of H-2D<sup>d</sup> (*B*) compared to Ly49C recognition of H-2K<sup>b</sup> (*C*). For clarity, all potential subsite-C residues are shown as ball and stick in both *B* and *C* but only those residues involved in recognition for the MHC I/Ly49 ligand pair are colored red on MHC I and mauve and lime green in the  $\beta$ 4- $\beta$ 5 and  $\beta$ 2- $\beta$ 2' loops of Ly49, respectively.

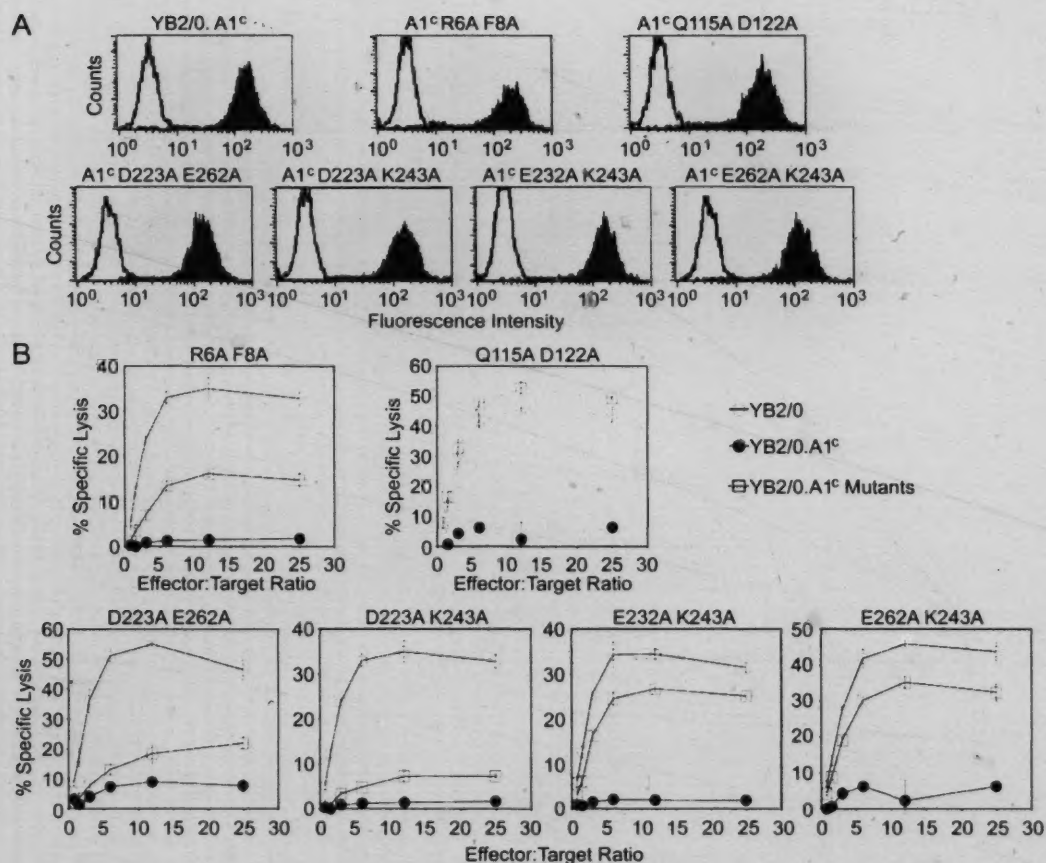


**Figure 4-8.** Double mutants at each subsite show that Ly49i2 recognition of RT1-A1<sup>c</sup> is completely dependent on subsite-F, less so on residues at the B-subsite and occurs in a unique manner at the C-subsite. *A*, YB2/0 cells expressing double mutants of RT1-A1<sup>c</sup> at the B- (R6AF8A), F- (Q115AD122A) and C-sites (D223AE262A, D223AK243A, E232AK243A and E262AK243A) were matched for surface expression to YB2/0 cells expressing wild-type RT1-A1<sup>c</sup> (shaded histograms) compared to untransfected YB2/0 cells (open histograms). *B*, YB2/0 cells expressing B-, F- and C-subsite double mutants of RT1-A1<sup>c</sup> were assayed for recognition by Ly49i2 expressing RNK-16 cells compared to untransfected or wild-type RT1-A1<sup>c</sup> expressing YB2/0 cells in 4-h cytotoxicity assays. Data represent the mean of triplicate wells  $\pm$ SD and are representative of three independent experiments.

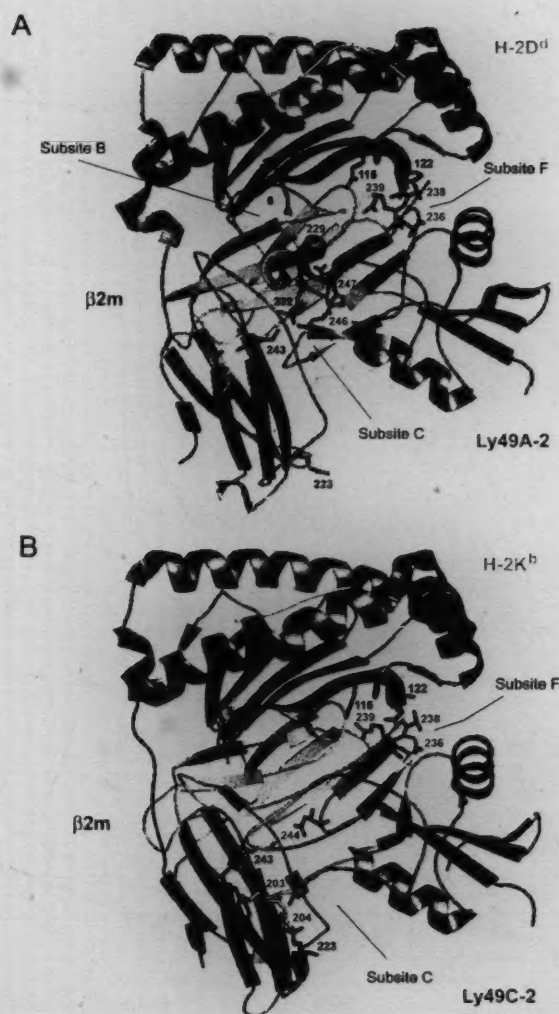


**Figure 4-9.** Location of key residues discussed in this study that were previously predicted to mediate recognition in the Ly49A/H-2D<sup>d</sup> and Ly49C/H-2K<sup>b</sup> co-crystals. MHC I residues that are important for Ly49A recognition of H-2D<sup>d</sup> (A) and Ly49C recognition of H-2K<sup>b</sup> (B) and correspond to residues mutated in this study are red; the conserved Ly49 triad consisting of residues 236, 238, 239 are orange;  $\beta 4$ - $\beta 5$  loop residues are violet;  $\beta 2$ - $\beta 2'$  loop residues are lime green and L3 loop residues are yellow. Residues on MHC I are numbered in black, those on Ly49 in green.

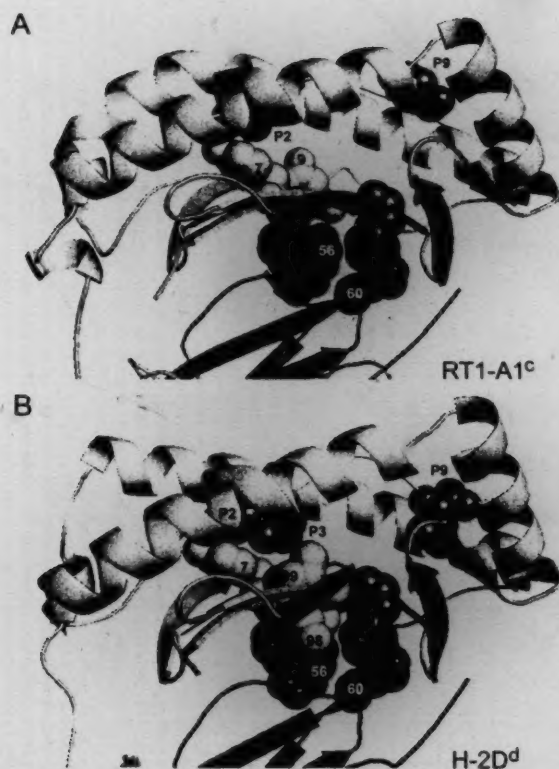


**Figure 4-10.** Polymorphisms in the anchor binding pocket(s) of MHC I may affect Ly49 recognition through conformational changes in solvent exposed residues that articulate simultaneously with the anchor binding pocket(s) of MHC I and with conserved  $\beta 2m$  residues that affect its orientation on MHC I. *A* and *B*, Solvent exposed residues of RT1-A1<sup>c</sup> directly articulating with the peptide anchoring B- and F-pockets of MHC I (*A*) and those of H-2D<sup>d</sup> directly articulating with the peptide anchoring B/C- and F-pockets of MHC I (*B*) all articulate with the conserved F56 and W60 residues that align  $\beta 2m$  on MHC I. Peptide anchor residues (*violet spheres*), B- and C- pocket residues (*grey spheres*). Residues articulating with the B- (*red*), C- (*yellow*) and F- (*orange*) pocket and conserved  $\beta 2m$  residues (*blue*) are depicted as spheres on the MHC I heavy chain (*grey*) and  $\beta 2m$  molecule (*blue*), respectively.



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## CHAPTER V

### DISCUSSION AND CONCLUSION

#### A. Summary of Contributions

Allele specific Ly49 recognition of MHC I has been documented in mouse (1-4), rat (5-9) and xenogeneic (2, 10-17) systems. Previous extensive mutagenesis (18-24) and co-crystallization studies (25, 26) isolated the Ly49 interaction at what is known as site 2 on mouse MHC I. Unfortunately, this site involves highly conserved solvent exposed residues below the peptide-binding groove on all three heavy chain domains and on the  $\beta 2m$  and provides no immediately identifiable means of mediating allele specific recognition by Ly49. While studies identifying polymorphisms between Ly49 receptors partially explained why Ly49 demonstrate allele specificity for their MHC I ligands (2, 26-30), it remained unsolved for some time how polymorphisms in Ly49 could distinguish between mouse MHC I alleles at a recognition site that is so highly conserved. To address this problem, we isolated a xenogeneic rat MHC I ligand, RT1-A1<sup>c</sup>, for the mouse inhibitory Ly49G and activating Ly49W receptors (31). In addition to being an interesting and useful finding for the study of NK cell involvement in xenotransplantation, the identification of a xenogeneic ligand allowed us to identify a motif on MHC I, shared between rat and mouse, which mediates recognition by these receptors. What we found to be a common motif between ligands was a similarity of supertype, with MHC I ligands for Ly49G, W and i2 receptors fitting best into the B7-supertype family, binding proline as an anchor at the P2 position of peptide within the B-pocket of the MHC I molecule, or in the case of the H-2D<sup>d</sup> ligand, binding glycine at P2 and proline at P3 within its double anchoring B- and C-pockets. In contrast, non-ligands for Ly49G and W were all of a different supertype with distinct anchor residues.

This was an interesting finding as a great deal of previous work on mouse Ly49 interaction with mouse MHC I highlighted the significance of the  $\alpha 1\alpha 2$  domains of the MHC I molecule in mediating recognition (32-34). The swapping of a recognized MHC I allele's  $\alpha 2$  domain for an  $\alpha 2$  domain from a non-recognized allele signified the importance of this domain in mediating interactions (35-37), an observation that was later



confirmed by co-crystallization of H-2D<sup>d</sup> with Ly49A (25). Mutagenesis studies also implicated the peptide-binding domains in mediating interactions, including residues within the peptide-binding groove (18, 20), but no one was ever able to establish precisely how the conformation of the  $\alpha 1\alpha 2$  domains and how polymorphic residues buried within the peptide-binding groove, which made no direct contact with the Ly49 receptor, were able to affect allele specificity. It wasn't until we identified the similarity of supertype between allelic MHC I ligands for Ly49G and Ly49W that a possible mechanism by which the residues buried within the peptide-binding groove of the  $\alpha 1\alpha 2$  domains, in the supertype-defining pocket, could be mediating Ly49 allele specificity for MHC I (31). This finding suggested that the allele specific recognition of MHC I by Ly49 receptors of innate immunity was intrinsically linked to structures important to adaptive immunity and implicated NK cells in immune surveillance for cells not just capable of expressing MHC I but expressing MHC I of a recognizable supertype.

Upon the identification of the natural syngeneic rat Ly49i2 receptor for RT1-A1<sup>c</sup> by Naper et al. (5) we were then able to demonstrate, not only in a xenogeneic rat/mouse system but also in a syngeneic rat system, that alterations specifically within the polymorphic and supertype-defining B-pocket of RT1-A1<sup>c</sup>, and not in non-anchoring pockets within the groove, could disrupt recognition (38). We also suggested a mechanism for how this could be occurring. Through comparison of crystal structures of ligands vs. non-ligands for Ly49G, W and i2 we found that the conformation of conserved solvent exposed residues below and articulating with this anchor-binding and supertype-defining pocket were identical for ligands whereas non-ligands had very different conformations. It was possible then, that the side chain conformations of residues articulating with the B-pocket were being affected by polymorphisms in the pocket, making them more or less available for interaction with different Ly49.

Peptide elution profiles from the B-pocket mutants of RT1-A1<sup>c</sup> indicated that mutagenesis of the polymorphic anchor-binding pocket might affect recognition on two levels. First, by modifying the structure of the MHC I heavy chain making solvent exposed residues at site 2 more or less available for interaction with polymorphic Ly49 as just mentioned. Second, through conformational effects upon the binding of peptides with anchor residues of a different supertype, or of a less preferred anchor type, in the anchor-

binding pocket. This gave insight into previously contradictory work showing that Ly49A recognition of H-2D<sup>d</sup> was peptide dependent but not usually peptide specific (34, 39), while Ly49C recognition of H-2K<sup>b</sup> was both peptide dependent and specific (40). Our elution profiles suggested that the peptide dependence previously seen for Ly49A recognition of H-2D<sup>d</sup> (34) was likely due to the requirement for the presence of peptides having anchor residues of the correct supertype but no requirement for specific peptide sequence beyond these anchor residues. Furthermore, upon examination of the peptides used in the investigation of the peptide dependence of Ly49C recognition of H-2K<sup>b</sup> (40), recognition appears to have been disrupted due to the loading of peptides with both subtle and overt alterations in anchor residues. Therefore, our work provided for the first time, a viable mechanism by which the structure and peptide content of the  $\alpha 1\alpha 2$  domains of MHC I, specifically the anchor binding B-pocket, could be mediating allele specificity at a highly conserved interaction site. Additionally, by examining the interaction of Ly49i2 with RT1-A1<sup>c</sup>, we provided the first evidence that rat Ly49/MHC I interactions occur in a manner similar to mouse, taking place at the site 2 interface and relying on supertype motifs.

We then established that both mouse Ly49G recognition of H-2D<sup>d</sup> and rat Ly49i2 recognition of RT1-A1<sup>c</sup> indeed relied specifically on residues articulating with the polymorphic N-terminal anchor binding pocket(s) at what we called the B-subsite, supporting our hypothesis that the conformation of the polymorphic anchor-binding pocket affected the availability of the solvent exposed residues that articulate with it, thereby affecting allele specific recognition by Ly49. In addition to a reliance on the B-pocket, we then demonstrated that, like the Ly49A/H-2D<sup>d</sup> and Ly49C/H-2K<sup>b</sup> allele combinations, Ly49G/H-2D<sup>d</sup> and rat Ly49i2/RT1-A1<sup>c</sup> specifically rely on a conserved "anchoring point" involving residues articulating with the other supertype defining, but more conserved, C-terminal peptide anchor-binding pocket at what we called the F-subsite.

Further examination of residues required for recognition by these rat and mouse Ly49/MHC I allele combinations allowed us to demonstrate that Ly49 also exhibit altered dependency on residues in the  $\alpha 3$  domain of MHC I, at what we called the C-subsite. This supported previous work in our laboratory demonstrating that polymorphic residues

in the  $\beta 4$ - $\beta 5$  loop of Ly49 are important for both syngeneic (29) and xenogeneic (31) allele specific recognition of MHC I, with Ly49G, W and i2 all having the DcGK motif in this loop, which may contribute to the common recognition of RT1-A1<sup>c</sup> by these receptors. We were then able to propose that the variable placement of Ly49 on the  $\alpha 3$  domain of MHC I, and therefore the varied ability to interact with MHC I alleles due to polymorphisms in the interaction loops of Ly49, could be due to direct anchor pocket mediated conformational changes in the  $\alpha 1\alpha 2$  domains of MHC I (Fig. 5-1).

Upon examination of crystal structures, we observed that the  $\alpha 3$  domain and  $\beta 2m$  orientations vary between MHC I alleles and previous mutagenesis work demonstrated that the B-pocket can affect  $\beta 2m$  orientation (41, 42). This allowed us to theorize that not only could conformational changes in the polymorphic N-terminal anchor-binding pocket(s) of MHC I directly affect Ly49 placement but the variable orientation of  $\beta 2m$  on different MHC I alleles could also be having an effect (Fig. 5-1). That peptides could also affect recognition through positional effects in the  $\alpha 3$  domain and  $\beta 2m$  is suggested by the observation of Achour et al. (43) that identical alleles of MHC I, containing varied peptides, also demonstrate a variability of  $\beta 2m$  and  $\alpha 3$  domain orientation. This seemed to explain the similar sensitivity of Ly49i2 to changes in the B-pocket compared to Ly49G, while having reduced reliance on solvent exposed residues at the B-subsite. Since  $\beta 2m$  association with MHC I is influenced by residues in the B-pocket (41, 42) it is possible that mutagenesis in the B-pocket of RT1-A1<sup>c</sup> disrupted Ly49i2 recognition primarily through alterations in  $\beta 2m$  placement and to a lesser degree on conformational changes of solvent exposed residues at subsite-B. To this end, we provided for the first time a comprehensive model for how both mouse and rat Ly49 may mediate allele specificity at the highly conserved site 2 of MHC I (Fig. 5-1).

## **B. Xeno-transplantation**

The limited supply of allogeneic organs for transplantation has made xeno-transplantation, specifically the transplantation of organs from animals specially raised for this purpose into humans, an attractive potential alternative. The immunological barriers to xeno-transplantation are formidable but studies involving the creation of



xenogeneic mixed chimeras have brought hope to the future success of xeno-transplantation without the need for long-term immunosuppression.

The creation of mixed chimeras involves the transfer of donor hematopoietic stem cells (HSC) to a host that has been made receptive through conditioning regimens involving the eradication or blocking of donor reactive lymphocytes and creation of "space" for the incoming donor cells without the eradication of the hosts own hematopoietic compartment. Both donor and host pluripotent hematopoietic stem cells can then contribute to the reestablishment of the host immune system post transplant. Studies demonstrate that successful chimerism involves the repopulation of the thymus with both donor and host APC capable of negatively selecting thymocytes that are reactive to donor or host epitopes. In this way, the production of a mixed chimera creates an immune system receptive to concurrent or consequent organ and tissue transplants from the same donor without the need for immunosuppressive drugs (44) (Fig 5-2A).

Conditioning regimens have been established in the study of xeno-transplantation of rat HSC and tissues into mouse. These regimens require the depletion of mature T cells from the donor HSC graft to prevent GvHD, depletion of both  $\alpha\beta$  and  $\gamma\delta$  T cells from the host to prevent rejection of the incoming HSC and both thymic (TI) and total body (TBI) irradiation to deplete xeno-reactive host cells and make "space" for incoming donor HSC (45). In contrast to allogeneic mixed chimeras, depletion of NK cells is additionally required for the successful engraftment of rat HSC in mice with greater chimerism being achieved in NK depleted recipients (45, 46). Although the original depletion of host NK cells prior to HSC transplant can facilitate successful engraftment and greater levels of chimerism (45, 46), longer-term chimerism and tissue graft acceptance appears impeded by the presence of newly developing NK cells incapable of recognizing inhibitory ligands on donor tissue (47) (Fig. 5-2A).

Previous studies suggested that xeno-reactivity between mouse activating Ly49 and rat MHC was responsible for NK cell mediated rejection (45, 48). This was due to examples of the activating Ly49D receptor recognizing targets from particular rat haplotypes (15, 16) along with the absence of a demonstrable mouse inhibitory receptor capable of mediating rat MHC I recognition (45). By finding that Ly49G recognizes the RT1-A1<sup>c</sup> MHC I allele of PVG rat (31), we are able to contribute to studies into the



ability of inhibitory Ly49, upon recognition of xenogeneic MHC I, to facilitate HSC and tissue graft acceptance in rat to mouse mixed chimeras. The presence of an inhibitory Ly49 receptor in mouse, capable of recognizing a rat MHC I molecule on rat donor HSC and tissue grafts, would potentially facilitate HSC engraftment, greater levels of chimerism and long term tissue graft acceptance without the need for NK cell depletion (Fig. 5-2B). Additionally, the recognition of RT1-A1<sup>c</sup> by mouse Ly49W (31) and the further identification of additional xenogeneic ligands through what is now a better understanding of the mechanisms mediating allele specificity, could facilitate the intelligent design of multi-ligand studies determining the ratio of activating to inhibitory signals required for optimal xenograft acceptance.

Clinically, the pig is considered the most likely candidate for xeno-transplantation into humans. Currently xenogeneic mixed chimerism experiments transferring pig HSC into primate recipients are under investigation, including investigation of concurrent or consequent tissue graft acceptance from the same donor (48). Although hyperacute rejection, primarily through pre-formed antibody recognition of  $\alpha(1,3)\text{Gal}$  moieties on porcine tissues (49), that are absent from primate tissues, is a major barrier to xeno-transplantation between these species, successful mixed chimerism has been shown to facilitate the tolerization of cells producing xenogeneic antibody (50, 51). Additionally, transgenic expression of human HLA-Cw3 on porcine tissues has proven to be a successful means of blocking human NK cell recognition of these xenogeneic targets (52), likely through engagement of inhibitory KIR, and could possibly eliminate NK cell mediated rejection of pig tissues transplanted into humans.

One wonders, considering our demonstration that Ly49 receptors share a similar reliance on supertype mediated conformations for recognition across species, whether species with similarly expanded KIR repertoires may also share similar reliance on supertypes or other pan-species motifs for recognition. Previous work has demonstrated that KIR may too rely on structures related to supertypes (53, 54), and previous demonstrations of reciprocal KIR recognition of MHC I between human and chimpanzee (55) suggest that xenoligands could potentially exist in pig since they also express an expanded repertoire of KIR receptors. Finding such a ligand in pig would be an enormous advancement in porcine to human mixed chimeras and xeno-transplantation, potentially

eliminating the costly requirement of transgenic HLA expressing pigs as sources of HSC and tissue grafts.

### C. Orphan Ly49 Receptors

The importance of allele specific inhibitory Ly49 receptors in the elimination of cells incapable of presenting peptide/MHC I to CTL, due to the down-regulation of MHC I upon viral infection or cellular transformation, is well established. Early work with mouse Ly49 receptors focussed mainly on identifying different Ly49 genes and identifying their allelic specificity for MHC I (3, 4, 56-58). While only one ligand has been identified for a rat Ly49 a number of mouse Ly49 ligands are known. Despite this, many, particularly activating Ly49, remain either orphan receptors or are found in strains where an activating MHC I ligand appears absent, leaving their role in innate immunity unresolved. Understanding the mechanism by which allele specific Ly49 recognize MHC I may facilitate the identification of potential ligands for these Ly49, such as viral MHC I homologues or rat ligands within the extensive RT1-C/E/U region, and help to better delineate the role of these receptors in innate immune responses.

#### *Viral Ligands*

A number of viral resistance loci map to the NKC including in CMV infection, *Cmv1*, 3 and 4, in herpes simplex infection, *Hrl* (59) and in ectromelia infection, *Rmp1* (60). Work with HSV has identified a NKC encoded non-Ly49 gene as potentially mediating this resistance (59) while *Clr* homologues in ectromelia (and rat CMV) may be responsible for NKC mediated (*Rmp1*) resistance to these viruses through *Nkrp1* receptors, since *Clr* have been shown to be the natural ligands for these receptors in mouse (61). To date only *Cmv1* has mapped to the Ly49 cluster, with CMV resistance being mediated by activating Ly49H receptor recognition of the CMV encoded viral MHC I homologue, m157 (62). Different alleles of the inhibitory Ly49I receptor have also been demonstrated to differentially recognize m157, mediating susceptibility to the virus by strains expressing Ly49I alleles that can recognize this virally encoded MHC I-like "decoy" molecule. Additionally, not all Ly49H alleles have been found to recognize m157 (63) and many wild MCMV strains contain mutated m157 molecules (64).

Interestingly, F-pocket like motifs in m157 closely resemble those found in some mouse MHC I alleles whereas another MHC I homologue in MCMV, m144, shows little similarity at either anchor binding pocket, possibly suggesting why m157 can be recognized by Ly49 and that a non-Ly49 NK cell receptor may mediate recognition for m144. It appears then, that whatever mediates the m157 interaction and the observed Ly49 allele specificity for m157 may be similar to the mechanisms mediating Ly49 specificity for MHC I. Therefore, our now greater understanding of how Ly49 mediate allele specificity may assist in the identification of additional candidate viral ligands for Ly49 receptors in rat and mouse. For example, the *Cmv4* locus, recently identified in the new inbred PWK strain derived from wild mice, also maps very closely to the Ly49 region of the NKC. Although the PWK NKC haplotype appears similar to that of C57BL/6 mice, this strain does not mediate resistance to MCMV through activating Ly49H recognition of m157. Since at least seven other MHC I like genes have been identified in murine and rat CMV, the PWK strain may encode a novel activating Ly49 for another MHC I homologue in MCMV thereby mediating resistance (64). Our now greater understanding of the mechanisms mediating MHC I allele specificity by Ly49 may assist in identification of this ligand pair.

Ly49 mediated resistance to viruses does not appear to solely rely on direct interaction with a virally encoded MHC I homologue. Desrosiers et al. (65) demonstrated that the Ly49P<sup>MA/My</sup> activating receptor could acquire specificity for H-2D<sup>k</sup> upon infection of H-2D<sup>k</sup> expressing targets with MCMV, possibly through the binding of H-2D<sup>k</sup> to virally derived peptides. This is supported by our findings and those of Franksson et al. (40) that Ly49 recognition of MHC I may be affected by the bound peptide. Although we demonstrated loss of recognition associated with alterations in the dominantly bound anchor residue, this implies that alteration of preferred anchor residues could also induce gain of function. This may be the reason why highly homologous activating and inhibitory Ly49 pairs exist, where very small polymorphisms between pairs could cause loss of inhibitory recognition and but gain of activating recognition of the same MHC I allele upon changes in the bound peptide repertoire upon infection or transformation.

Viral MHC I homologues are also found in human viruses, and include UL18 in HCMV (66) and MC080R in *molluscum contagiosum* (67). While direct binding of UL18



occurs through the inhibitory LILRB1 (68) and not KIR, KIR receptors are possible candidates for interaction with MC080R and other MHC I homologues. Additionally, both activating and inhibitory KIR show sensitivity to bound peptide with examples of inhibitory KIR recognizing a specific HLA supertype only in the context of peptides from Epstein-Barr virus (54). Additionally, the presence of the activating KIR3DS1 with HLA-Bw4 is associated with delayed progression to Acquired Immunodeficiency Syndrome (AIDS) (69) suggesting this activating KIR may also recognizes MHC I alleles preferentially in the context of viral peptide. Therefore, the identification of virally encoded or induced ligands for Ly49 receptors may facilitate understanding of and serve as models for human KIR interactions with viruses.

#### *Rat Ly49 Ligands*

The Ly49 repertoire in the rat has only recently been extensively characterized (70-72). Despite many historical clues involving ALC experiments that point towards the presence of activating and inhibitory rat Ly49 pairs that functioned similarly to mouse Ly49 in the allele specific recognition of MHC I (9, 73-78), only RT1-A1<sup>e</sup> has been demonstrated as a discernible MHC I ligand for one rat receptor, Ly49i2 (5). At least 25 functional rat Ly49 have been described to date with many receptors demonstrating allelic differences between strains similar to mouse Ly49 (2, 27, 70). Close homology of rat Ly49 to mouse Ly49 and the demonstration that rat Ly49i2 mediates recognition and allele specificity for RT1-A1<sup>e</sup> similarly to how mouse Ly49 recognize MHC I alleles indicates that rat receptors may in general similarly rely on subsites at site 2 and supertype-defined conformations to mediate allele specificity for MHC I ligands. This suggests that scrutiny of the anchor binding pockets and peptide anchor preferences of rat MHC I could identify potential candidate ligands for rat Ly49. The pre-selection of candidate ligands for activating rat Ly49 would be of particular use as rat activating receptors have typically been implicated in the recognition of MHC Ia-like Ib molecules in the RT1-C/E/U region (76, 79), which encodes a much larger repertoire of potential ligands than is found in the mouse (80), and would otherwise make ligand identification difficult and time consuming.



The examination of anchor binding pockets for the identification of ligands is most likely to assist in the identification of RT1-A ligands that are preferentially recognized by rat inhibitory Ly49 (74, 78, 81-83) since they are the major loci recognized by CTL and are therefore most likely to be of recognisable supertypes. Despite this, examination of MHC Ia-like Ib molecules in the rat indicate that many bind peptide and  $\beta 2m$ , have similarly conserved  $\alpha 3$  domains, can act as ligands for CTL (80, 84, 85) and therefore may also be discriminated between by Ly49 via recognizable supertype motifs. Additionally, preliminary alignments of RT1-C, E2, E, U and the recently characterized Ia-like RT1-L MHC Ib molecules (86) indicates that despite reduced overall polymorphism in these molecules compared to the RT1-A loci, anchor binding pocket polymorphism is found within RT1-C, E and U alleles. Therefore anchor-binding pocket conformation may also act as determinants of Ly49 allele specificity for these Ib molecules at the conserved site 2. Considering the importance of *rattus norvegicus* as a widely used model for human disease and transplantation studies (87, 88), the linkage of the RT1-C/E/U locus to many of these diseases (89-91) and the involvement of NK cells (92-94), it would be of great profit if our findings describing Ly49 discrimination of MHC I alleles could aid in the identification of additional RT1 ligands for rat Ly49.

#### **D. Convergent Evolution**

Convergent evolution is the independent evolution of the same motifs at multiple times in evolution. Such convergently evolving motifs tend to be selected for based on structure or function and not sequence (95). For example, the independent expansion of the structurally and sequentially unrelated KIR and Ly49 receptors in two distinct chromosomal gene complexes in primate and rodent lineages, respectively, appears to be an example of functional convergent evolution to produce NK cell receptors capable of monitoring MHC I expression in an allele specific manner. In contrast, when comparing the anchor binding pockets of MHC I alleles of a similar supertype, but from different species with no apparent MHC I orthologues, one finds that although the residue sequence lining the anchor binding pockets of each molecule is divergent, that the overall structure of the pocket is nearly identical and is an example of structural convergent

evolution between species to produce peptide binding pockets capable of binding particular supertype-defining peptide anchor residues.

That Ly49 receptors, examples of convergent evolution themselves, appear to have evolved to recognize supertype-defined motifs on MHC I that also appear to be a product of convergent evolution, leads one to consider what the selective driving advantage might be. With our findings that a rat inhibitory Ly49, which is non-orthologous to mouse Ly49G and W, but similarly depends on supertype-defined motifs in a rat MHC I molecule that is also non-orthologous to mouse MHC I, raises the possibility that the convergent evolution of superotypes in these two species may also be the driving force behind the convergent evolution of rat and mouse Ly49 receptors that recognize supertype-defined conformations. Finally, since KIR are the functionally equivalent receptors in humans to Ly49 receptors in rodents, it is worthy of consideration that the same evolutionary forces driving Ly49 expansion, diversification and allele specific recognition in rodents may similarly play a role in the expansion, diversification and function of KIR.

#### *Ly49 Receptor Surveillance and CTL Function*

T cell receptor recognition of MHC I is dependent on two main factors, the recognition of the peptide by TCR residues primarily in the complementarity determining region (CDR) 3 loops of the TCR and MHC I recognition primarily by the CDR1 and CDR2 loops of the TCR (96). This combined requirement of peptide in the context of a recognizable "self" MHC I allele for TCR recognition is known as MHC restriction. The TCR interacts with MHC I directly from above, sampling both the bound peptide and residues on the  $\alpha 1\alpha 2$  domains of MHC I and is typically oriented diagonally across the top of the peptide binding groove so that the CDR loops in the  $\alpha$  chain of the TCR are directly over the B-pocket, and the  $\beta$  chain CDR loops are interacting with residues nearer to the F-pocket (97) (Fig. 5-3). Although co-crystals of MHC I and TCR complexes show variability in the exact angle of orientation of the TCR and usage of residues in MHC I for interaction, CDR1 $\alpha$  and CDR2 $\alpha$  loops interact predominantly with residue 65 in the  $\alpha 1$  helix and to a lesser degree with residues 62 and 158 (Fig. 5-3) while the CDR1 $\beta$  and CDR2 $\beta$  chains interact primarily with residue 155 in the  $\alpha 2$  helix and to a lesser degree

with residues 69, 72, 150 and 151 (96). Although there has, to date, been no demonstrated reason for this fairly conserved TCR/MHC I docking arrangement or basis for MHC restriction it may be related to MHC I supertypes.

Because the CDR1 and CDR2 loops of TCR are generated from V gene segments and are less diverse compared to the highly variable CDR3 loops that are made up of V, J and, in the  $\beta$  chain, D $\beta$  segments that contact peptide and because MHC I and TCR genes are unlinked, it may be possible that MHC restriction is a driving factor behind the convergent evolution of MHC I supertypes to maintain a repertoire of about nine or 10 conformations recognizable by TCR despite the high degree of MHC I polymorphism that permits the binding of a diverse peptide repertoire. This possibility is supported by evidence that syngeneic recognition appears to rely primarily on contributions from the CDR1 $\alpha$  and CDR2 $\alpha$  loops that typically interact with  $\alpha$  helix residues affected by the supertype defining B-pocket (98) (Fig. 5-3). Interestingly, TCR recognition of xenogeneic MHC I also appears to rely predominantly on the B-pocket associated loops (99) raising the possibility that xenogeneic TCR interactions, like Ly49 xenogeneic recognition of MHC I, are possibly mediated through MHC restriction motifs related to shared supertype conformations across species. That MHC I supertypes may be important for MHC restriction is further supported by experiments showing that even very minor alterations in the peptide binding groove can affect CTL recognition of an otherwise identical peptide, possibly due to conformational changes in the MHC I domains mediating restriction (100, 101). Additionally, although viral escape mutants of exposed peptide residues can abolish direct CDR3 recognition of peptide, alterations in buried peptide residues can also affect the conformation of the  $\alpha$ 1 $\alpha$ 2 domains and TCR recognition potentially through altered MHC restriction (102).

Supertype motifs appear important for successful adaptive immune responses, whether they are important for MHC restriction or not (103). The similar frequency of different supertypes across ethnicities, despite the highly variable allele representation within each supertype (104, 105), indicates that there is strong selective pressure to maintain nine or 10 supertype conformations, presumably for immune fitness against a battery of pathogens. That there are some identical supertypes in species as divergent from humans as rat and mouse could be explained by similarities in the peptide loading



machinery in all three species that produces peptides with C-terminal residues typical of proteasome cleavage patterns (106) or P2 residues produced by the cleavage patterns of aminopeptidases (107). That there is not a completely overlapping repertoire of supertypes in rodents and primates may also be partially explained by differences in the peptide loading machinery, for example the presence of the TAP-A allele found in rat that preferentially transport peptides into the endoplasmic reticulum with positively charged C-terminal residues (108), may allow for a different supertype containing a charged residue in the F-pocket of rat MHC I that would not be possible in mice since they have a TAP incapable of transporting this type of peptide. Partially overlapping supertype repertoires in divergent species could also reflect the species-specific evolution of TCR genes to recognize immunodominant epitopes in species specific pathogens, resulting in CDR1 and 2 loops requiring a potentially different, but still limited, repertoire of species-specific supertypes. It would be interesting to examine the peptide elution profiles from the MHC I of geographically distinct wild rats or mice for the presence of a limited repertoire of species-specific supertypes amid extensive allelic polymorphism.

The presence of innate immune receptors in both mouse and rat, which appear to mediate allele specificity based on supertype motifs found in MHC I, is understandable based on the apparent importance of supertypes for immune fitness. That mouse and rat Ly49 can detect the presence of supertype conformations may ensure that cells incapable of binding a specific repertoire of immunodominant peptides in the context of a recognizable self MHC supertype for MHC restriction, and therefore TCR recognition, will be cleared by NK cells. Inhibitory Ly49 may specifically bind MHC I alleles bound to self-peptides or pathogen derived peptides that are of specific supertypes and therefore recognizable by CTL. Upon prolonged infection or transformation this bound repertoire may derive escape mutants. Although some escape mutants include peptides altered such that they no longer mediate direct TCR recognition through the CDR3 loops (109), escape mutants are also seen with alterations in buried and supertype-defining peptide residues (102, 110). Alterations in buried peptide residues may abolish structures important for MHC restriction resulting in loss of CTL recognition but this may also disrupt supertype conformations recognized by inhibitory Ly49, allowing NK cell



clearance of infected cells presenting these mutants. In support of this possibility, the peptides used in the experiments by Franksson et al. (40) that disrupted Ly49C recognition of H-2K<sup>b</sup> were all derived from viral sources and contained slight alterations in anchor binding residues. Such altered peptides may also result in slight alterations in conformation such that activating Ly49, highly homologous to the previously engaged inhibitory receptor, gain recognition for a slightly altered supertype structure.

Genetic expansion is a common feature of pathogen resistance genes (111, 112). The evolution of highly polymorphic MHC that maintain nine or 10 supertype conformations may allow organisms that evolve much more slowly than their infecting pathogens to "keep up" with pathogens while maintaining a minimal number of structures capable of mediating MHC restriction. The expansion of the Ly49 receptor repertoire is thought to be driven by the polymorphic nature of their MHC I ligands (113) but this was previously irreconcilable with the knowledge that Ly49 interact with a highly conserved interaction site on MHC I. If polymorphisms defining supertype-defined conformations on MHC I are responsible for mediating Ly49 allele specificity, then supertype motifs may also be mediating Ly49 gene expansion. The evolution of receptors that can distinguish between MHC I supertype motifs would be an efficient way for non-rearranging receptors such as Ly49 to differentiate between an extremely large repertoire of alleles expressed from an unlinked locus.

KIR may also have some reliance on supertype motifs since mutations in the B-pocket of HLA-B7 disrupted KIR recognition (53) and KIR3DL2 appears to recognize HLA of specific superotypes (54). Despite this, the interaction site between KIR and HLA does not occur near the B-pocket but nearer to the F-pocket although P7 and P8, not P9, residues are critical for KIR recognition of HLA alleles (54, 114, 115). Despite these apparent contradictions, KIR interaction near the TCR CDR2 $\beta$  interaction site with MHC I and KIR may have evolved to survey for MHC restriction motifs at the TCR V $\beta$  interaction site in combination with peptide.

The apparent convergent evolution of rat and mouse Ly49 to recognize MHC I alleles at site 2, which involves not only residues articulating with the supertype-defining pockets of the  $\alpha$ 1 $\alpha$ 2 domains but also with residues in the  $\alpha$ 3 domain and  $\beta$ 2m, suggests that these receptors may have an additional role in surveying for MHC I capable of

interacting with the co-stimulatory CD8 molecule that acts in concert with the TCR for optimal activation of CTL. Indeed, Ly49 receptors and CD8 share a number of residues in common for recognition of MHC I (116) and loss of residues critical to CD8 binding may also disrupt protection through inhibitory Ly49, thereby eliminating cells with defective ability to interact with CD8. In contrast, human KIR do not interact at this site and cannot survey for MHC I capable of interacting with CD8. Instead, LILRB1 that interacts with a number of MHC I alleles through their conserved  $\alpha 3$  domain and  $\beta 2m$ , appears to mediate this function in human systems (117). Interestingly,  $\beta 2m$  in murine species contain conserved residues critical for Ly49 recognition that are not found in the  $\beta 2m$  of species that have not expanded the Ly49 locus (24). Additionally, mouse  $\beta 2m$  has been shown to have a uniquely flexible S4 strand compared to human and it is this strand that contains residues that articulate with the supertype-defining pockets of mouse MHC I, resulting in the variable orientation of  $\beta 2m$  between MHC I alleles (118). That mouse and not human  $\beta 2m$  contains this flexible motif resulting in allelic variation in  $\beta 2m$  positioning on MHC I, may add credence to our model that murine Ly49 rely on variable positioning of  $\beta 2m$  in the mediation of allele specificity whereas human KIR mediate allele specificity at a site distinct from  $\beta 2m$ . Whether rat shows this similar flexibility of  $\beta 2m$  between MHC I alleles, or alleles loaded with variable peptides, will require the examination of numerous MHC I alleles in this species and could provide additional support for our model.

#### *Functional Divergence in Rodents*

Mouse and rat inhibitory Ly49 may have convergently evolved to recognize MHC I alleles based on supertype-defined conformations for the surveillance of MHC I capable of being recognized by MHC restricted TCR. It is unclear though, whether activating rat Ly49 receptors, which appear to predominantly recognize MHC Ia-like Ib molecules in the rat RT1-C/E/U, will be similarly restricted by supertype structures since only a limited number of Ib molecules have been demonstrated to present peptide to CTL (80, 84, 85). Some MHC Ib molecules in the rat have been shown to be expressed at reduced levels compared to MHC Ia due to the usage of promoter sequences similar to those found in the mouse H2-Q and H2-T Ib genes (86) that can be upregulated by paracrine

mediators (119). Therefore, rat activators may only come in to play upon upregulation of their ligands due to paracrine indicators of infection. This is in contrast to mouse activating Ly49 that appear to overcome dominate inhibitory signals upon paracrine stimulation without visible alteration in their ligands' density (120). There is also the possibility that rat activating Ly49 may recognize some MHC Ib molecules solely in the context of pathogen derived peptides through peptide induced  $\alpha 1\alpha 2$  domain conformations that determine Ly49 specificity. This of course is all speculative until more specific ligands can be identified for rat Ly49 and their ability to mediate CTL recognition is determined.

Rat and mouse Ly49 additionally appear to have diverged somewhat in their usage of residues at the Ly49 236, 238, 239 triad that interacts with residues at the F-subsite of the MHC I molecule, seemingly anchoring the Ly49 into position. This could be due to the presence of two TAP alleles in the rat that can load a more diverse repertoire of residues into the F-pocket of MHC I and therefore potentially cause more conformational variability at the F-subsite. Additionally, the inability of identical single residue alterations in MHC I to disrupt recognition by Ly49i2, compared to Ly49G and other mouse alleles, indicates that rat Ly49 while similarly binding site 2 and relying on supertype conformations in the  $\alpha 1\alpha 2$  domains, may rely more heavily on different residues at site 2 for recognition. If this is a consistent finding in rat Ly49 it will be further evidence for convergent evolution between Ly49 receptors of these two species to produce receptors capable of recognizing supertype motifs and MHC I capable of interacting with CD8, but relying on different overall orientations and/or residues in Ly49 to mediate this recognition.

While most of the possibilities mentioned here are somewhat speculative, they are worthy of consideration based on information both we and others have gathered and could form the basis for future work in the field to define the specific roles of, and mechanisms behind, Ly49 surveillance of "healthy" and "unhealthy" MHC I in two evolutionarily divergent rodent species.

### **E. Future Directions**

While theories on convergent evolution can never be proven, many of the possibilities that our work on Ly49 allele specificity raises are indeed readily testable. These include such topics as the role of Ly49 in the surveillance of recognizable supertypes for CTL function, the roles of peptide and  $\beta 2m$  in mediating allele specific recognition and further characterization of rat Ly49 and their ligands. Additionally, the identification of a xenogeneic rat MHC I ligand for mouse Ly49 receptors allows further investigation into the role of NK cells in xenogeneic mixed chimerism.

#### *Xeno-transplantation Studies*

The possibility that natural xenogeneic MHC I ligands on donor HSC and tissues may mediate NK cell tolerance through recognition by allele specific donor Ly49 or KIR (depending on the species) is possibly best first examined through the ability of mice naturally expressing either the inhibitory Ly49G or activating Ly49W to reject interperitoneally introduced RT1-A1<sup>c</sup> expressing mouse derived tumour cells either more slowly or quickly, respectively, compared to identical but non-RT1-A1<sup>c</sup> expressing tumour cells. Additionally, it would be interesting to determine if a mouse concurrently expressing inhibitory Ly49G and activating Ly49W would demonstrate dominant inhibition toward RT1-A1<sup>c</sup> expressing tumour targets.

Harvesting HSC from PVG rats that naturally express RT1-A1<sup>c</sup> for implantation in Ly49G expressing BALB/c mice receiving a xenogeneic pre-conditioning regimen that does not include depletion of host NK cells, would determine if the Ly49G expressing subset in BALB/c is capable of facilitating acceptance of RT1-A1<sup>c</sup> expressing HSC and the generation of high levels of mixed chimerism in the absence of NK cell depletion. Although the initial subset of Ly49G expressing NK cells in BALB/c mice may be too small to facilitate the high levels of mixed chimerism seen with NK cell depletion regimens, NK depleted host mice should be assessed to see if the expression of RT1-A1<sup>c</sup> within a PVG/BALB/c mixed hematopoietic compartment would stimulate the production of a larger repertoire of Ly49G<sup>+</sup> NK cells through the stochastic expression of *Ly49* genes that appears to occur until a receptor capable of recognizing a "self" MHC I



ligand, RT1-A1<sup>e</sup> in this case, is expressed (121-124). BALB/c mice chimeric for PVG donor HCS could then be assessed for their ability to mediate long-term tolerance to subsequent RT1-A1<sup>e</sup> expressing tissue grafts compared to secondary grafts in traditional mixed chimerism experiments that use NK cell depletion instead of xenogeneic recognition to mediate NK tolerance.

As more xenogeneic ligands are identified, possibly through our now better understanding of mechanisms mediating allele specificity, mixed chimeras could be designed to determine what percentage of the NK cell repertoire needs to express an inhibitory receptor vs. activating receptors for the greatest induction of mixed chimerism and long-term tissue graft acceptance without immunosuppression. Xenogeneic ligands for KIR may also be found through closer examination of MHC I motifs shared between other KIR expressing species, through conservation of sequence or convergent evolution, allowing similar studies to those we propose in rat and mouse to occur between porcine and primate subjects that may apply to clinical studies in the future.

#### *Peptide Loading Studies*

Our finding that the-supertype-defining pockets of MHC I affect allele specific recognition by Ly49 generates additional questions that need to be answered. First, it would be interesting to examine the ligand repertoires of other Ly49 to see if they too fit into supertype groupings or if specificity relies on residues exclusively in the polymorphic anchor binding pocket of the MHC I. Additionally, complete "swapping" of anchor binding pockets between differentially recognized MHC I alleles to see if it results in a concurrent "swapping" of supertype, defined by peptide elution experiments, and transfer of Ly49 specificity from one MHC I allele to the other would lend further support to our model.

Second, since mutations deep in the B-pocket of RT1-A1<sup>e</sup> created a shift toward the binding of secondary anchor residues or anchor residues of a completely different supertype in the B-pocket of these mutant molecules, it would be interesting to artificially load peptides with variable anchors to cells expressing 'empty' RT1-A1<sup>e</sup> molecules and assess for their ability to mediate recognition by Ly49. Such an experiment would involve surface cleavage of endogenous RT1-A1<sup>e</sup>, proteasome inhibition and cellular

growth at 26°C to permit assembly of RT1-A1<sup>c</sup> molecules at the cell surface that are receptive to added peptide. Since Orihuela et al. (34) were able to demonstrate Ly49A recognition of H-2D<sup>d</sup> loaded with the minimal anchor residue containing AGPAAAAAL peptide, it would be interesting to assess if minimal peptides capable of similarly stabilizing RT1-A1<sup>c</sup> at the cell surface but containing either dominant, secondary or even non-typical residues at the P2 anchor position results in similar, reduced or disrupted Ly49 recognition. Such experiments would need to include the addition of murine serum instead of foetal calf serum to ensure that artefacts due to exchange of murine for bovine  $\beta$ 2m on MHC I did not obscure results as they may have previously for Ly49A recognition of H-2D<sup>b</sup> (21, 125). Such artefacts are due to the absence of Ly49 interaction residues in bovine  $\beta$ 2m and may include the potentially non-flexible nature of bovine  $\beta$ 2m in adopting the supertype and peptide mediated orientations that are seen in mouse and not human MHC I structures (118). Should such peptide loading experiments result in variable levels of Ly49 recognition it would be valuable to produce crystal structures (or Ly49/MHC I co-crystal structures where possible) of MHC I folded with minimal peptide to analyse if differences in the  $\beta$ 2m and  $\alpha$ 3 domain orientation could explain the change in recognition. Furthermore, the creation of more co-crystals of variable MHC I/Ly49 allele combinations, starting with H-2D<sup>d</sup> and H-2D<sup>k</sup> with Ly49G, would allow us to assess more directly the differences in  $\beta$ 2m and  $\alpha$ 3 domain orientation and Ly49 docking orientation compared to the co-crystals that are already in existence.

The activating Ly49P receptor, which is highly homologous to Ly49A but only recognizes H-2D<sup>d</sup> and not H-2D<sup>k</sup> under normal physiological conditions, can gain recognition of H-2D<sup>k</sup> upon MCMV infection (65). It would be interesting to elute peptides from both healthy non-recognized and recognized MCMV infected H-2D<sup>k</sup> expressing targets for comparison of the dominant anchor residues found in each scenario. Should a pattern be evident, similar loading experiments such as those described for RT1-A1<sup>c</sup> recognition in the previous paragraph, could provide the first evidence of a pathogenic peptide repertoire-induced gain of recognition by an activating Ly49 receptor. Additionally, assessment of whether H-2D<sup>k</sup>/MCMV peptide structures mediating Ly49P recognition simultaneously result in loss of Ly49A recognition would support the theory that homologous activating receptors gain recognition of their

homologous inhibitory counterpart's ligands upon pathogen-induced alterations of the peptide repertoire.

Finally, viral escape mutants have been identified for murine TCR that involve alterations in peptide residues buried within the peptide binding groove and inaccessible to the TCR (102, 126). It would be interesting to assess whether such escape mutants, particularly those involving residues within main or secondary anchor binding pockets, also result in the loss of recognition by inhibitory Ly49, or gain of recognition by their homologous activating counterparts. Such experiments would test the hypothesis that Ly49 receptors survey for changes in MHC I conformations that affect TCR recognition, particularly those pertaining to supertype-defining pockets.

### *Rat Studies*

While we have produced the first, and extensive, analysis of the molecular mechanisms mediating rat Ly49 allele specific recognition of a rat MHC I molecule, there are many questions remaining to be addressed in this species. Although we unequivocally show that rat Ly49 interact with MHC I at site 2, the fact that we were unable to totally disrupt recognition of RT1-A1<sup>c</sup> by Ly49i2 through the same single mutants that disrupt mouse Ly49 recognition of MHC I, suggests that rat Ly49 may either be of greater affinity for MHC I, or that they have a slightly altered reliance on key residues for recognition at site 2. Biacore studies comparing the binding constants of rat Ly49i2 with RT1-A1<sup>c</sup> could determine if this Ly49, and perhaps rat Ly49 in general, interact with their ligands with greater affinity. Elimination of the extensive glycosylation found on Ly49i2 compared to mouse Ly49 by generation of these molecules in a bacterial system may obscure results and production of wild-type Ly49i2 and glycosylation mutants from a mammalian pituitary cell line would likely yield more interesting and physiologically relevant results. Additional mutagenesis studies may define more critical residues mediating rat Ly49 recognition at site 2 and clues to these residues may be gleaned from examination of the slightly altered Ly49 236, 238, 239 triads found in rat Ly49 that interact at the F-subsite of MHC I and the variable  $\beta 2$ - $\beta 2'$  and  $\beta 4$ - $\beta 5$  loop sequences found in rat Ly49 that may differentially interact with conserved residues in the  $\alpha 3$  domain. Additionally, although little is known of the interaction between rat Ly49

and  $\beta 2m$  the presence of the conserved K3 and Q29 residues found solely in rodent  $\beta 2m$  molecules and the altered orientation seen between the two known allele structures of RT1-A (127) hints that like mouse, rat Ly49 may too rely on residues in  $\beta 2m$  and  $\beta 2m$  orientation for recognition. While mutagenesis of  $\beta 2m$  residues implicated in mouse Ly49 recognition would determine whether rats and mice similarly rely on residues in  $\beta 2m$  for Ly49 recognition, co-crystal structures of Ly49i2 and RT1-A1<sup>e</sup> and other rat allele combinations as they are identified, would be of greatest value in understanding the role of  $\beta 2m$  orientation in rat and mouse systems.

The discovery of more rat Ly49/MHC I allele combinations, possibly with the aid of our findings describing the motifs mediating recognition, and the development of additional rat Ly49 and MHC Ib specific antibodies will facilitate additional functional experiments in this species similar to those already described for mouse and with Ly49i2 and RT1-A1<sup>e</sup>. Such experiments could include describing whether rat Ly49 that recognize MHC Ib do so in the context of supertype surveillance for CTL recognition, as may be the case for mouse and rat Ly49 recognition of MHC Ia, or if recognition is mediated solely by up-regulation of Ib ligands during infection. Elution of peptides from MHC Ib molecules in the rat may also define a role for Ly49 in recognizing MHC Ib molecules solely in the context of pathogenic peptide.

## **F. Conclusion**

By identifying a xenogeneic MHC I ligand for mouse Ly49, we were not only able to contribute to the field of Ly49 allele specificity but also to the field of xenogeneic mixed chimerism in studies of transplantation. Additionally, we map in detail for the first time, the interaction site mediating rat Ly49 recognition of rat MHC I. Not only have we clearly demonstrated the role of the polymorphic anchor-binding and supertype-defining B-pocket in mediating allele specific recognition we have also demonstrated variable reliance by Ly49 on residues at site 2 and have been able to propose a viable and comprehensive mechanism, substantiated by examination of previously published work and our own findings, mediating allele specific interactions between Ly49 and MHC I both in mouse and rat systems.



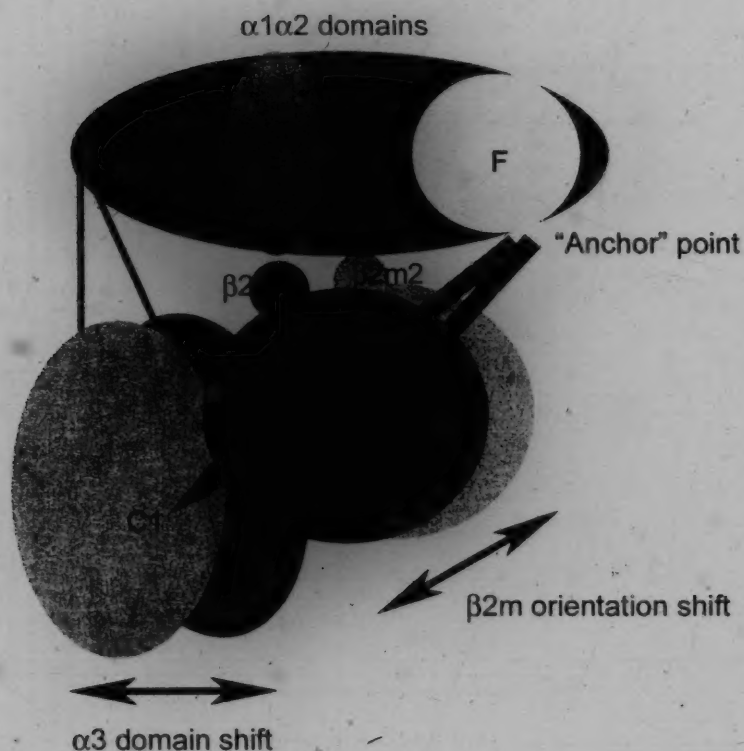


**FIGURE 5-1.** Cartoon depiction of how conformational changes in the  $\alpha 1\alpha 2$  domains, particularly in the supertype-defining B-pocket, may alter MHC I recognition by allele specific Ly49 at a highly conserved interaction site. Polymorphic anchor binding pockets (B1 and B2) articulate with  $\beta 2m$  altering its orientation on MHC I ( $\beta 2m1$  and  $\beta 2m2$ , respectively) and may also affect the orientation of the  $\alpha 3$  domain (C1 and C2, respectively). The conserved 236, 238, 239 triad in Ly49 anchors receptors at subsite F (yellow) but Ly49 are differentially placed at subsite B and C due to differential  $\beta 2m$  and  $\alpha 3$  domain orientations. Ly491 has polymorphic loops capable of interacting specifically with the MHC I allele 1 orientation while Ly492 has the polymorphic loops required to interact specifically with the MHC I allele 2 orientation.

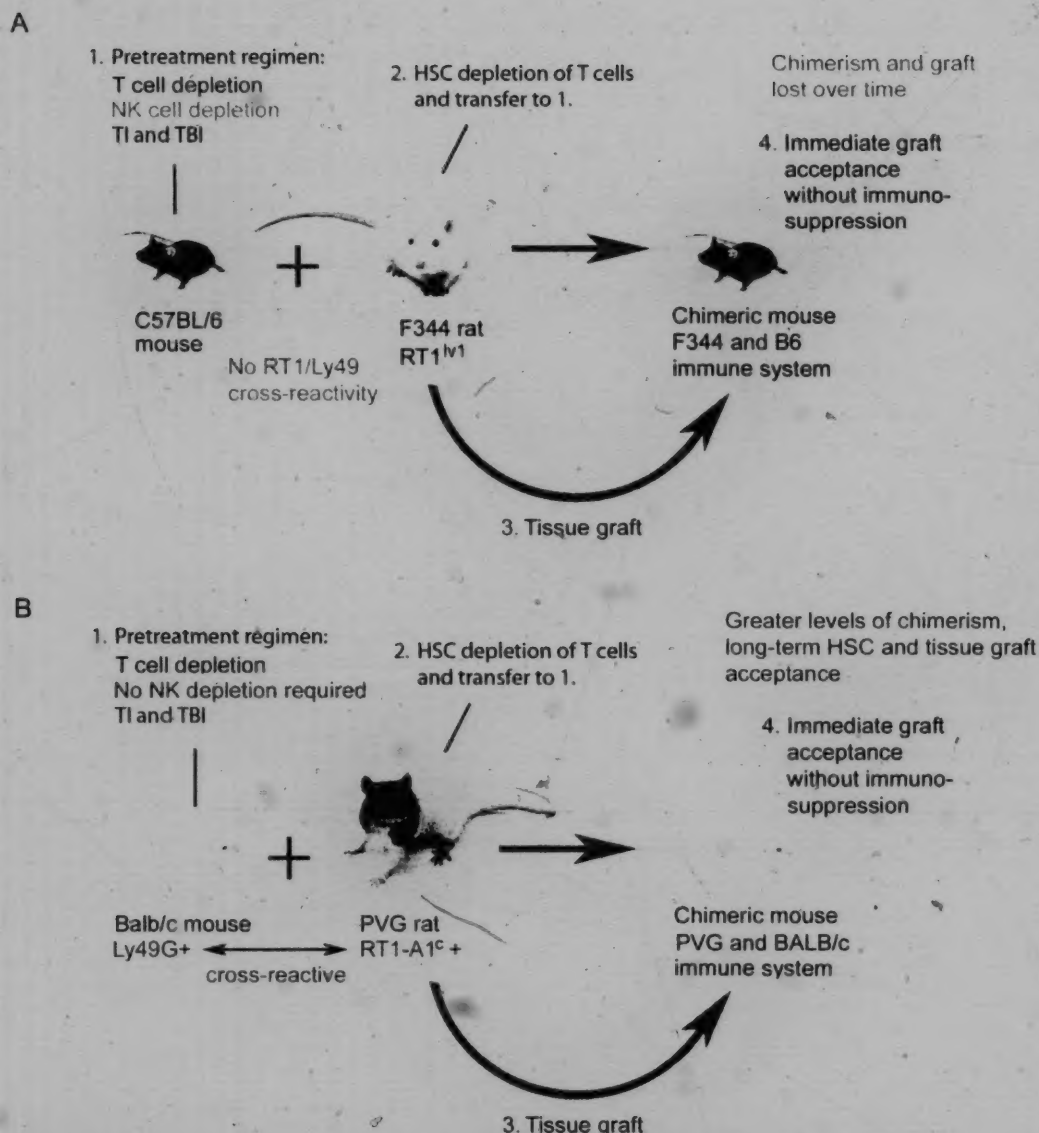


**FIGURE 5-2.** Creation of xenogeneic mixed chimeras. *A*, Generation of mixed chimerism using mouse strains that do not express an inhibitory Ly49 capable of interacting with rat MHC I is possible by using NK cell depletion in the pre-transplant regimen but does not result in long term graft acceptance or high levels of chimerism likely due to re-emergence of NK cells incapable of recognizing MHC I on donor tissues. *B*, The expression of inhibitory Ly49G in BALB/c mice may induce NK cell tolerance and facilitate greater levels of chimerism and long-term tissue graft acceptance from PVG donor rats through recognition of the RT1-A1<sup>c</sup> MHC I molecule on rat cells without the need for NK cell depletion.

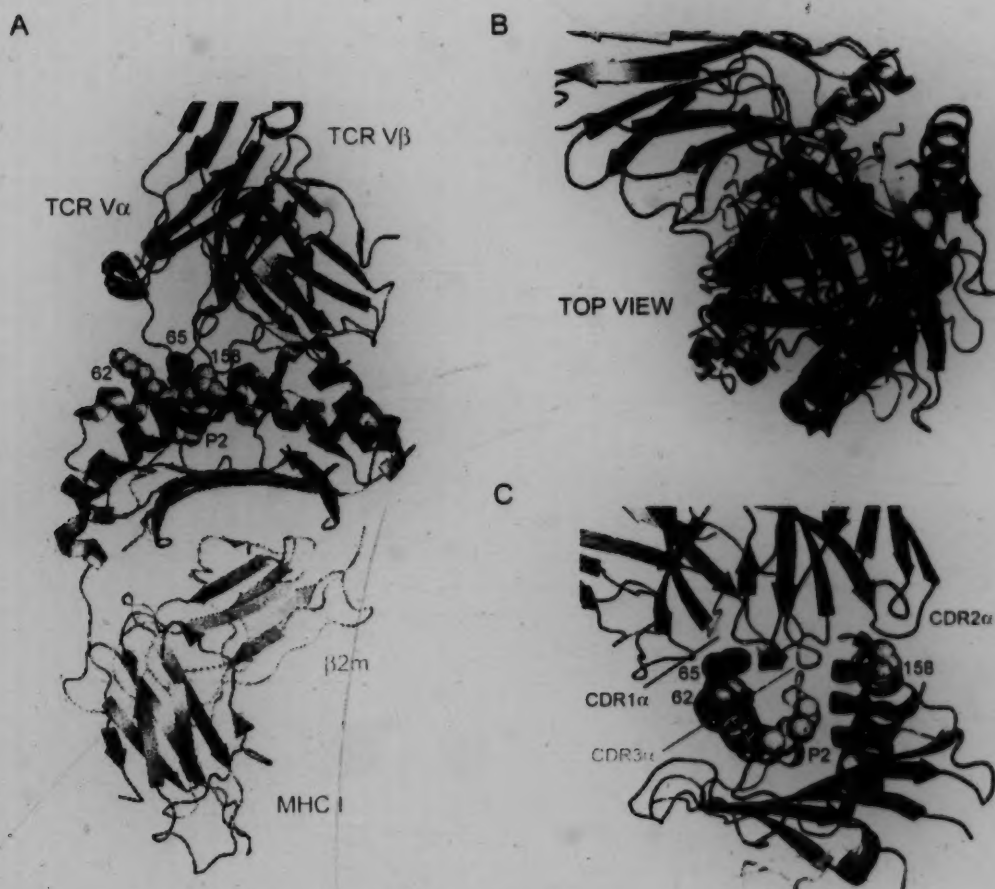


**FIGURE 5-3.** T cell receptor (TCR) interaction with MHC I. *A*, Side view of the TCR interaction with MHC I (slate blue) showing how it contacts the  $\alpha 1\alpha 2$  domains from above with the TCR V $\alpha$  domain (red) interacting with residues associated with the P2 peptide anchor-binding B-pocket (pink spheres). *B*, Top view of the TCR interacting with MHC I showing the TCR V $\alpha$  domain (red) position directly above the B-pocket. *C*, Transverse view along the length of the peptide-binding groove showing how the CDR1 $\alpha$  (navy) and CDR2 $\alpha$  (purple) loops predominantly interact with B-pocket associated residues in MHC and the CDR3 $\alpha$  (orange) loop with peptide (yellow). Only the V $\alpha$  (red) and V $\beta$  (green) domains of TCR are shown.  $\beta 2$ -microglobulin ( $\beta 2m$ ) is shown in grey. The dominant TCR interaction residue in MHC I is depicted in dark pink.

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